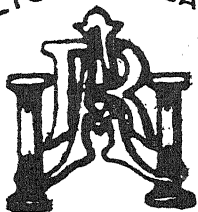


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THE PHYSIOLOGICAL SIGNIFICANCE OF WEBER'S  
LAW AND COLOUR CONTRAST IN VISION.

By J. S. HALDANE.

It was originally shown by Bouguer [1760] that over wide limits the degree of visibility of a shadow thrown by a local obstruction to a source of light depends, not on the intensity of the illumination produced by the light, but on the ratio between the illumination of the surface on which the full illumination falls, and the illumination of the shadow. The visibility of the shadow thus depends on this "geometrical" ratio, and is not proportional arithmetically to the difference between the illumination of the shadow and that of the surface illuminated by the light. He also found that, as has often been confirmed, the smallest difference which can be appreciated steadily in this ratio is about 1.5 p.c. In his recent book on *Photometry* Walsh [1926] puts this figure at 1.6 p.c., as a result of experiments at the National Physical Laboratory with the improved form of the Lummer-Brodhun photometer.

Weber [1846] showed that a similar conclusion applies to other kinds of sensory stimuli. It thus came to be known as Weber's Law of Sensory Stimuli, and to be regarded as a fundamental law covering response to differences in sensory stimuli. It was later taken by Fechner and other psychologists as a fundamental psychophysical generalization covering the supposed relation of body to mind. I propose in this paper to discuss, in the light of further experiments, the physiological significance of Weber's Law as applied to visual sensation, including its extension to colour sensation.

It is known that under certain conditions—for instance with extremely feeble illuminations—the law ceases to hold; but I wish first to point out usual conditions where, even with strong illuminations, it does not hold at all, and the visibility of a shadow depends simply on the arithmetical difference between the illumination on a shaded and on an illuminated surface.

The experiments described in this paper have been to a large extent carried out with the help of the simple form of Rumford or Lambert shadow photometer which I recently designed for testing rapidly the

candle-powers of miners' lamps [1928, 1933]. With this photometer the shadow of a standard light is thrown by an upright bar about  $\frac{1}{4}$  in. wide on an upright white screen, and a shadow of the lamp to be tested is thrown side by side with it on the same screen, the shadows being thrown at a very acute angle to one another, and being looked at from above and directly in front. The lamp is then moved with one hand along an ordinary photometer scale (with graduations numbered proportionally to the squares of their distances from the shadow) till the shadows are equal in depth. If the standard light is the usual one (for miners' lamps) of one candle-power, the reading on the scale, with the standard light in its ordinary fixed position, gives directly the candle-power of the lamp, and would be 1.00 on an average if the lamp were exactly of one candle-power. We are comparing the illuminations of two white surfaces, each of which is illuminated by one of the lamps; and by varying the candle-power of the standard, or its distance from the screen, we can easily verify the fact that in accordance with Weber's Law the minimum steadily visible difference in illumination is, within wide limits, a fraction of about 1.5 p.c. of the illumination given by either lamp, as was originally pointed out by Bouguer.

But now let us throw a very feeble shadow, from a distant or very feeble light, across the white surface and top of the strong shadow from one near light. It will be seen that this feeble shadow is no more easily visible on the strong dark shadow than on the strongly illuminated white surface. Thus Weber's Law does not hold at all in this case. A difference which, when measured with the photometer, can be shown to be about 1.5 p.c., is just visible on the strongly illuminated white surface; but on the strong shadow, where the percentage difference in illumination caused by the feeble shadow is immensely greater, the feeble shadow is not more visible, provided that the strong shadow is not very wide. As we widen this shadow, however, the weak shadow becomes relatively more and more visible on the strong shadow.

We can easily show that the diminished visibility of the weak shadow on the stronger one is due to the surrounding illumination of the white screen by the near lamp. If we cut off this illumination, the feeble shadow at once stands out as a very strong one, in accordance with Weber's Law. In ordinary daylight the general field of vision is strongly illuminated, and we can now see that as regards differences of illumination within details of this field, Weber's Law does not hold at all. It is easy to verify this by experimenting with a feeble shadow, like that on the photometer screen. Under ordinary circumstances, therefore, it is not

geometrical but arithmetical differences in illumination that we appreciate. The latter are what we are accustomed to call the "objective" differences. The conditions under which Weber's Law can be verified are quite special; and when we realize this we can see the need for a much wider physiological generalization than is embodied in Weber's Law.

If we balance the shadows of two lamps on the photometer, we can easily, without direct interference of any kind with the shadows, upset the balance seriously by placing a piece of black paper on one side of the screen. The effect is to make the nearest shadow relatively brighter; and a difference of as much as 20 p.c. in the relative brightness of the two shadows may be produced, as measured with the photometer, if the black surface reaches as far as the shadow nearest to it. This experiment shows how necessary it is, in using the photometer, that the two shadows should be well in the centre of a uniform field. It has also been familiar to me for long that in using a colorimetric method (for instance with a Gowers-Haldane hæmoglobinometer), the tube on one side commonly appears darker than that on the other, owing to differences in illumination between the two side backgrounds. Hence it is necessary to transpose the tubes at each observation; and colorimeters, however elaborate, which do not permit of this may give rise to much error.

Although Weber's Law does not hold under ordinary conditions, a fact which does hold is that within wide limits of general illumination in the physical sense, the visibility of shadows, and consequently of the illuminated objects which we see, remains practically the same. When the illumination becomes very feeble, this generalization no longer holds. If, however, we observe more closely, we find that in darkness so complete that we cannot see any objects at all, the field of vision is nevertheless illuminated by what is known as the "intrinsic" illumination or light. This intrinsic illumination can be seen, when the eye has adapted itself to the darkness, to consist of minute and evanescent areas of light and darkness, producing the general impression of grey, with considerable apparent brightness, which, if we judge by the contrast between dark and bright within it, seems to be about the same as that of the average field of vision in daylight. We can now realize that in very feeble illumination external objects disappear because the illumination from them has become a physiologically inappreciable fraction of the intrinsic illumination, and that in less feeble illumination objects are indistinct because the intrinsic illumination has partly covered up the differences in their own illumination.

In reality we are in the presence of a very fundamental physiological fact with regard to vision. This is that though from a physical standpoint the external light acting on the eye varies throughout enormous limits, from zero to that of full daylight, which is about 100,000 times greater than the illumination by which on an average an English coal-miner works at present, the physiological illumination of the field of vision remains, so far as can be judged from the contrast of brightness with darkness, about the same. Brightness of bright areas, and darkness of dark areas, are relative always to a general field of visual activity which, apart from the temporary effects of sudden change in illumination, is persistent. What appears as light or dark is thus an expression of contrast within this field. When we pass rapidly from what, in the physical sense, is bright illumination to dim illumination, physiological after-effects of the bright illumination remain for some time, adding to the intrinsic illumination; also, as is well known, fading in intensity and often producing changes in colour. Hence time elapses before vision of surrounding objects becomes "adapted" to dim light—in other words before such vision becomes possible as the unreinforced intrinsic illumination permits.

Before further discussion I shall refer to experiments on colour-vision. It is well known that though, from a physical standpoint, the quality of daylight, light from incandescent filaments or mantles, and from various kinds of flame, varies very considerably, yet what we call a white surface seems, or may seem, about equally white in light from any of these sources. The phenomena of coloured shadows, the existence of which has been known for centuries, appear, however, to reveal in a most striking manner the differences between the different qualities of light; and in using a photometer, when the relative brightnesses of different kinds of light are being compared, the differences in colour of the shadows are very prominent. In reality coloured shadows reveal a great deal more than colour differences, as will now be shown.

If we allow a shadow produced by the light of a candle to fall on the photometer screen, and beside it a shadow produced from daylight coming through a narrow chink left open in a shutter, or from an electric lamp with blue glass giving about the same quality of light as daylight (a "daylight" lamp), the shadow thrown by the candle will, as is well known, appear blue, and that thrown by the daylight orange-yellow, while the rest of the screen appears white. One shadow is illuminated by the daylight alone, and the other by the candle alone. If the two shadows are not equally bright, one has a dark, and the other a light



tint; but if we now adjust the two lights (by varying the distance of the candle or in some other way) so that the shadows are of about equal brightness, the one shadow will have a very surprising deep blue colour, and the other a deep orange-yellow colour, though in spite of the difference in colour we can, with a little practice, estimate the relative brightnesses quite well. The colours are equally evident even if the shadows are widely separated from one another, and they are quite clearly due to simultaneous contrast.

We can vary this experiment in various ways. For instance, we can substitute for daylight the light from an electric light with a red glass. The shadow illuminated by the candle alone will appear as a deep green, and the other shadow as a deep red. If we use a green electric lamp the shadow illuminated by the candle alone will appear as a deep red, and the other as a deep green. With an orange electric lamp, the shadow illuminated by the candle will be dull blue, and the other shadow yellow-brown. With a lemon-yellow electric lamp the shadow illuminated by the candle becomes dull violet. For the candle light we can substitute the almost pure spectral light from a sodium flame, but the result is much the same. We can make the surface illuminated by the candle or sodium flame or daylight any tint we please, from red to blue or violet, by varying the quality of the other light. To anyone seeing these experiments for the first time they are most striking and surprising. With the help of a suitable lantern screen the whole of them can be shown to a large audience. The earliest, and still in many ways the best, description of coloured shadows produced experimentally is that of Rumford [1794], and he writes with enthusiasm about them.

It is now evident that the colour of an illuminated object does not depend simply on the physical quality of the light reflected from it; and indeed every artist paints as if he knew this. Coloured shadows and similar contrast effects produce a grossly exaggerated or completely distorted impression of the "physical" differences in different kinds of light. Coloured shadows are somewhat of a bugbear in photometry, not so much because it requires some practice to distinguish differences in intensity of illumination from differences in colour, as because with different colours different intensities are not quite the same for different individuals. Very striking quantitative data illustrating this fact are given in a recent paper by Dudding, Winch and Cooper [1931].

By covering the screen with non-reflecting black paper, except at the place occupied by the shadows, we can cut off any influence of the surrounding illuminated and apparently white surface. This does not

alter the colours seen except that they both appear brighter, and the shadows now appear as illuminated surfaces. The colour effect, therefore, depends in this case on the contrast between the two shadows. If, however, we separate the two shadows widely, and then block out one of them from the field of vision, the colour of the visible shadow is unaltered, so that its colour is due to contrast between the shadow and the surface, illuminated by both lights, around it. When the brightness of the shadows is balanced the surface has double the illumination of either shadow; but one half of this is due to the illumination on the other shadow, and to the remaining half must be attributed the fact that the contrast effect of the apparently white surface on a shadow is the same as that of the other shadow alone.

If we cut off the light entirely from one of the lamps, the shadow which is left appears simply black, while, if the light illuminates a sufficiently large white surface, at some distance, this surface may appear almost white, though the lamp itself, and any white surface close to it, appears strongly coloured. If, now, we make the shadows very broad, we can see that the contrast effect is most marked close to the edges of each shadow, and diminishes with distance from them. The contrast effect is thus a partly localized one.

We obtain a similar result if, with the help of a black diaphragm, we illuminate one half of a large white screen with ordinary electric light, and the other with blue or red electric light. When the intensities of illumination are about the same the part illuminated by the ordinary electric lamp appears as yellow or green, particularly near where the two illuminations meet, and the other half as blue or red; but with much stronger illumination by ordinary electric light the corresponding part of the screen becomes of a lighter and the other half of a darker tint.

We can vary the experiments in another manner. We can cut a hole in the photometer screen, and then arrange a source of light falling on it so that the light passes obliquely through the hole, and does not fall on any visible part of a white surface placed behind. This surface is, however, so arranged that it is illuminated by a chink of daylight, or a concealed "daylight" lamp, arrangements being also made so that the intensity of the latter illumination can be varied by altering its angle of incidence on the illuminated surface or in some other way. By this means we can vary the brightness of the surface seen through the hole without altering the brightness of the light falling on the screen. What can now be seen is very striking. Let us suppose that the screen itself is illuminated by an ordinary electric lamp, and thus appears white.

The surface seen through the hole will now appear blue. But the more the illumination of this latter surface is reduced, the deeper does the blue become, while the more its illumination is increased, the lighter does the blue become. If, now, when the surface seen through the hole in the screen appears deep blue, we turn off the electric light illuminating the screen, the surface seen through the hole becomes rapidly pure white. The blue is of course the complementary colour to the yellowish electric light, but all the time the surface of the screen appears pure white. If we throw coloured light on the surface beyond, its colour is twisted round towards blue.

Instead of illuminating the screen with ordinary electric light we can use light of any other quality. The screen itself will appear more or less white, while the surface seen through the hole will have the complementary colour to that of the light; and the less brightly the surface seen through the hole is illuminated, the deeper is its complementary colour. Only when the surface beyond begins to be more brightly illuminated than the screen does the complementary colour begin to appear round the hole.

We can also vary the experiments in a negative manner by cutting out the influence of the illumination surrounding what we are looking at. This can be done by looking with one eye through a narrow tube, blackened inside, and at the same time taking care that no light enters the eye at the near end of the tube. A tube about a centimetre in internal diameter and 30 cm. long is convenient, and can easily be made by rolling up a sheet of dull black paper, preferably lined inside with black velvet, and gumming it in position. Blackened tubes were first used by Rumford [1794] for experiments on coloured shadows.

If, now, we look through the tube at the orange-red shadow thrown by daylight or a daylight lamp on the photometer screen, we see that it rapidly fades to a much less intense form of yellow, similar to that given with the tube when the candle alone illuminates the screen. It is the same with all the varied tints of coloured shadows produced when we substitute for daylight various coloured lamps. We have eliminated the contrast effect between the tint of the candle illumination and that of the screen. We get a similar result with the shadow cast by the candle. The heightened colour of this shadow fades into the white or much less intense colour seen through the tube when the screen is only illuminated by the daylight or coloured lamp.

It is also the same when we look at the variegated colours seen through the hole in the screen in the experiments described above when

the surface behind is illuminated by a daylight lamp. They all fade into white or something very near it when the hole is looked at through the blackened tube, though the colours appear again if the tube is so held that part of the screen comes into view. In actual fact, the "daylight" lamps supplied to me give, in spite of their blue glass, a distinctly blue shadow with ordinary daylight; and a white surface illuminated by the lamp looks, through the tube, distinctly yellow.

We can now see a physiological explanation of a phenomenon which has always been more or less of a puzzle—namely the blueness of the sky. If we take the blackened tube and look up at a piece of clear blue sky, the colour soon fades into pure white, just as in the experiment in which the hole in the screen is looked at through the tube. But the blue reappears again if we allow a white cloud to come into the field of vision, just as when we allow part of the screen to come into the field of vision in the experiment with the hole in the screen. The light coming from the clear blue sky has much less of the violet end of the spectrum cut off than light coming through clouds or obliquely from the sun, since the violet end is more readily scattered by particles in the air. Physically speaking, therefore, the light coming through clouds, or obliquely from the sun, is yellower than that from the clear sky. Even a clear sky looks blue, since all surrounding objects illuminated by the sun have their colours twisted round, like the colour of the clouds, towards white. Through a blackened tube this effect is undone, so that white becomes yellow, blue changes towards white, green towards yellow, red towards orange, and purple towards red.

It is well known that when the sun is low, and particularly if it is shining through rather misty air, it looks red, and throws a corresponding green shadow on a white surface illuminated from the sky above. When the sun is higher and looks yellow, the shadow is blue or violet; whereas with the sun high up the shadow becomes black. There is no difficulty in explaining the coloured shadows as simultaneous contrast effects, but when we turn to the blue colour of the sky it seems at first difficult to explain this as a contrast effect, since brightly lit clouds look quite white, or colourless. When, however, we examine these clouds, or a white surface lit by them, on an overcast day, with the blackened tube, we can see that they gradually turn yellow, which is not the case with the clear overhead sky.

But if the clouds are "really" yellow, which they appear to be when the disturbing effects of the peripheral field of vision are eliminated in the blackened tube experiment, why do they not look yellow? And why,

in general, do white surfaces look white when they are illuminated by yellow electric light, or still more yellow candles? Why, also, do they look more or less white when they are even illuminated by red, or green, or blue lamps? When any of these surfaces are examined with the blackened tube they appear in what might be regarded as their "real" yellow, red, green, or blue colours. This very pertinent question will be discussed presently.

A very striking phenomenon, which can often be observed in tropical countries with a clear sky as the sun is just disappearing at sunset, or appearing at sunrise, is known as the "green ray." The light coming directly from the disappearing or appearing upper edge of the sun appears for two or three seconds of a brilliant green colour. One of Jules Verne's novels takes its title from this appearance. In a recent paper Lord Rayleigh [1930] pointed out that the light in the violet half of the sun's light must be more refracted by the atmosphere than the light in the red half, and hence must be visible longer at sunset, and earlier at sunrise. He also devised an experimental arrangement which enabled this effect to be studied at leisure. The colour seen was, however, blue, not green. It now seems evident, in view of the experiments described above, that the cause of the actual sunset or sunrise coloured ray being vividly green, is the simultaneous contrast effect due to the light coming from round the sun being red.

When, with the ordinary coloured shadow experiment we keep the relative intensities of illumination of the shadows the same, we see their tints equally distinctly over a very large variation of illumination, and if we make both shadows very faint by removing both sources of light to a distance, or in any other way, the difference in colour remains still visible so long as the two shadows are separately visible. It is thus relative, and not absolute differences in illumination that determine the visibility of the colours in the shadows. We can, therefore, to judge from this experiment, extend Weber's Law to colour appreciation when we regard each colour and its complementary colour as similar in its physiological behaviour to brightness and darkness.

On the other hand, this extension of Weber's Law appears as if it were quite unjustifiable when we consider the fact that when colours are produced locally by pigments, each colour appears the same regardless of what other colour is adjacent to it. We can, for instance, place red and yellow patches together without the yellow being turned green, whereas in the coloured shadow experiment yellow light becomes green.

We are at once reminded of the fact, already discussed in connection

with uncoloured shadows, that although, within wide limits, the visibility of a shadow depends, not on the intensity of general illumination, but on the contrast between the general illumination of the neighbouring surface and that of the shadow, yet when a shadow is produced locally without sensible alteration of the general illumination, the visibility of this shadow depends directly on the (arithmetical) difference between its illumination and that of the neighbouring surface. Similarly a difference in visible colour produced locally by altering the quality of the light illuminating a large surrounding or neighbouring surface depends, not on differences which can be arithmetically expressed between the two lights, but on the contrast between the colour actually given by one of the qualities of light and its complementary colour given by the other; yet when the visible colour is produced locally without sensible alteration of the general illumination, the colour depends directly (arithmetically) on the difference in quality between the illumination given by the surface and that given by a white surface under the same general illumination, or, failing illumination from without, by the intrinsic illumination. It appears, therefore, that we must interpret the presence or absence of colour contrast in the same way as presence or absence of contrast with bright and dark illumination.

In accordance with this statement, coloured shadows possess tints which are complementary to one another in so far as they are tinted at all. But patches of colours produced by pigments in the same general illumination have a tint which is independent of the co-presence of other patches of colour. In darkness, moreover, a luminous patch, such as that produced by a coloured lamp, has what we are accustomed to call its "natural" colour.

The physical difference between a coloured surface illuminated by a white light and a white surface illuminated by a coloured light is that in the former case that part of the white light which by itself would produce a colour complementary to that of the coloured surface is largely absorbed, and the rest largely reflected, thus producing the colour seen. In the latter case nearly all the coloured light illuminating the white surface is reflected; so that the surface illuminated exclusively by each coloured light might be expected to appear of the same tint as the respective coloured light. In actual fact, however, the colour of neither surface may correspond to the light which is illuminating it, and the colours which appear may be complementary to one another.

The difference as regards presence or absence of contrast effect between a coloured surface produced locally by a pigment and one

produced by coloured light in a coloured shadow experiment depends on the facts already discussed. As is well known, however, to painters as well as physiologists, contrast effects may be visible in shadows thrown by ordinary daylight or other light of one quality, next to a coloured surface. In this case the contrast effect is not produced by two different kinds of light transmitted to neighbouring surfaces, but by the two different kinds of light reflected from two neighbouring surfaces. Contrast effects of this kind are, however, much less readily produced, and less striking than those produced by two different kinds of light transmitted to neighbouring white surfaces.

By throwing a shadow of suitable depth on a white background by the side of a pigmented surface we can obtain a distinct though rather feeble contrast effect, the colour of the pigment near the junction being deepened, and the complementary colour appearing in the nearest part of the shadow. Necessarily, however, the complementary colour appears dark as it is in shadow; and the deepening in the colour of the pigment is correspondingly slight. It is easy to produce this localized contrast effect by putting some tissue paper over a pigmented surface and a black surface next to it. A better method, as is well known, is to use a rotating disc for producing the darkened surface. In all these experiments it is necessary to distinguish carefully the simultaneous contrast which is characteristic of them from the successive contrast which is apt to be confused with it.

The explanation of the localized contrast effect at the edge of a shadow which is neither too light nor too deep is that though a local pigmented surface has no sensible effect on the general standard of colour appreciation in the field of vision, it has a distinct localized effect close to the edge of the coloured surface, thus producing a contrast effect which is noticeable when the intensities of illumination on the two contrasted surfaces are about equal, just as the far more prominent colour contrast of coloured shadows is most striking under the same conditions.

Without using lights of different colours we can produce the coloured shadow effect with a sufficiently large pigmented surface round a white surface, if we shut off other surrounding illumination, so that only the pigmented surface and sufficient of the white surface are visible. We can make the intensities of illumination on the two surfaces equal by inclining the white surface, so that it appears darker in accordance with Lambert's "cosine law." The contrast effect extends over the whole of the white surface, as in a coloured shadow experiment; but the result is not

so striking, since there is, for comparison, no surface which appears white.

The reason why the deep colours produced by pigments disappear in growing darkness before the coloured objects themselves disappear is the fact that the intensity of illumination of the colours in the pigmented objects is so much less than in white or light-coloured objects. Towards night all deeply pigmented objects become black.

Just as we can disturb the balance of depth of illumination on the photometer screen by introducing a black surface on one side, so also we can disturb balance of tint by introducing a coloured surface on one side. Thus if we produce on the photometer screen two shadows of the same tint, we can alter this tint by introducing a coloured surface on one side. As I showed many years ago, we can by tintometric titration with carmine solution determine with great accuracy the percentage saturation of hæmoglobin with carbon monoxide. Examples of this are given in a paper by Douglas and myself [1912]. But in comparing the tints of the blood solutions in the two test-tubes, differences in the colour of the background behind the sides of the two test-tubes have so distinct an effect that it is always necessary to transpose the positions of the tubes at each observation, just as with an ordinary colorimetric determination. A further point which comes out clearly in this method is that with different persons the amount of carmine which has to be added to produce equality of tint differs quite markedly, though the final result as regards percentage saturation is exactly the same. Similarly in photometric work, with a difference in colour between the two lights compared, there are, as already mentioned, appreciable differences between practised observers as regards the candle-power of the lamp. For some persons, for instance, intensity of light from a flame lamp as compared with that from an electric standard is definitely greater than for others.

#### DISCUSSION.

We can unify or explain the whole of the phenomena relating to Weber's Law in its relation to vision, and to coloured shadows and simultaneous contrast generally, if we assume that a normal constancy tends to be maintained in the field of visual activity, in such a manner that when one part appears brighter or coloured, the rest, and particularly what is immediately adjoining, tends to appear darker or of the complementary colour to a corresponding extent. Let us consider in detail how this theory covers the facts.

We may first take the facts which Weber's Law embodies. The



theory at once covers the fact that over an enormous range of general illumination in the physical sense the visibility of brightness and darkness remains practically the same, and that over a similar range of general illumination the visibility of complementary colour difference remains practically the same, although, as Purkinje discovered, the range is not quite the same for all colours (unless, as we have seen, they are complementary).

The theory also covers the fact that of whatever quality in a physical sense the general illumination may be, unpigmented objects, such as white paper, tend to appear as white. Except when we first come into it, or look at objects through a blackened tube, we are not even aware that ordinary electric light is yellow, and candle or other flame-light still yellower. Nor have we ordinarily any suspicion of the yellowness of the light on an overcast day. But even when a room is illuminated by a deep yellow, red, green, or blue electric lamp, the colour of white paper looks nearly white, and its deeply coloured illumination in a physical sense is only realized on looking at it through a blackened tube.

In the next place the theory covers the fact that in the details of what is visible as bright or dark in the field of vision, or as coloured, Weber's Law does not hold at all. Both degrees of bright and dark, and degrees of colour are seen in accordance with that we are accustomed to regard as the physical difference between them, and not as ratios between bright and dark, or between colour and complementary colour. This is at once intelligible when we consider that any mere local variation in the field of vision has, unless it is of great relative magnitude, almost no influence in disturbing the general balance of brightness, or colour in the visual field. In ordinary daylight it is the overwhelming influence of the general illumination which standardizes our vision of the details of brightness and colour. It is only when the sun's rays are falling very obliquely, and much less intensely, with a clear sky above, that we are aware of their yellowness or redness by contrast with the light coming down more or less vertically from the sky; but the fact that the clear sky, or light reflected from the sea, is blue, reveals the physical character of the yellow light from clouds and sun. The light from the blue sky is feeble as compared with the yellow light coming directly, but obliquely, from the sun, or through the medium of bright clouds. Hence it is the sky that looks blue, and not the sunlight or clouds that look yellow, just as in the experiment with a hole in the photometer screen.

With a relatively intense light in one part of the field of vision, the rest of the field is of course darkened, and an intense coloured light, if it

is localized by some means, turns the rest of the field of vision towards its complementary colour. This also becomes intelligible on the theory. The fact, on the other hand, that colour contrast effects due to ordinary differences in colour in small parts of what is visible and illuminated by the same light are localized at the junctions of the parts, and only visible clearly when the brightnesses of the two parts are about equal, is equally intelligible.

Finally, the fact that when we look at things through a sufficiently narrow blackened tube we see things in what for physics is their objective colour, becomes quite intelligible. White in the physical sense is such a mixture of light of different wave-lengths as gives the sensation of white. Our sensations correspond with those produced by this mixture when we practically shut out disturbing contrast effects by admitting to the eye only a very small and even part of such light shining from without in the ordinary field of vision. We get this sensation when we apply the tube to the pure "blue sky", or to a white surface illuminated exclusively by it, but by no means by looking through the tube at anything which may appear to us white without use of the tube.

In surrounding darkness we obtain a similar "objective" perception of the colours of small parts of the field of vision. We notice at once, for instance, that electric light is yellow, and we see coloured lamps in their "objective" colours, though we can easily change these colours into quite different ones by altering the surrounding illumination. If, for instance, we paste a piece of translucent paper over a hole in the photometer screen, and illuminate this paper by coloured light from behind the screen, we at once change the colour of the paper to white or to various other colours than that of the lamp behind, by changing the colour of a lamp illuminating the front of the screen.

When there is no light falling on the eyes, and any after-effects of previous illumination have ceased, intrinsic illumination remains, in accordance with the theory, and is simply a colourless grey, which in no way affects the colour of any localized illumination from outside. Hence by using a sufficiently narrow blackened tube, we can see what can be regarded as the "objective" colours of what we see, though these "objective" colours are simply the colours seen under certain physiological conditions, so that we have made no real separation between objective and subjective. If the tube is insufficiently narrow, this method of course defeats its own object, since a large illuminated surface will tend to appear white, whatever its "objective" colour may be. In using the blackened tube we must also be careful to avoid the influence of

successive contrast on the intrinsic illumination. If, for instance, after looking in the ordinary way at a coloured shadow, or at blue sky, we use a blackened tube in looking at it, we at first see the same colour, and a little time elapses before this colour changes into the "objective" colour. It is the same if we look at an apparently white surface which is illuminated by coloured artificial light or daylight from an overcast sky, or at a pigmented object illuminated in the same manner. Through the blackened tube they all take on only gradually the "objective" colour, the reason being that the after-effect on the intrinsic illumination takes some time to disappear, with the result that the colour, as well as the brightness, of what is seen through the tube is at first greatly affected by simultaneous contrast. In his discussion of coloured shadows Helmholtz [1867] was entirely deceived by this fact, which led him to interpret the blue colour of a candle shadow in presence of daylight as a psychological effect. This had the unfortunate effect of leading physiologists to neglect the very striking phenomena of coloured shadows, although Hering [1887] pointed out later that the blue colour seen through a blackened tube disappears rapidly. He attributed it, though not in a quite correct manner, to successive contrast.

This leads me to a discussion of successive contrast, which has hitherto been only incidentally referred to. On the theory which has been developed above, simultaneous contrast is an expression of the fundamental tendency in visual activity to maintenance of a normal general balance. Thus the physiological phenomena of brightness and darkness, and of colour and complementary colour, are part of the adaptation which is occurring everywhere in visual phenomena. But adaptation in any direction, once established, has a tendency to persist. Hence the intrinsic illumination, on looking through a blackened tube, is at first markedly affected by the previous illumination, with the result that the simultaneous contrast effect previously present remains for a short time. In darkness the after-images of previous visual excitations are positive, in accordance with this interpretation, although they may afterwards become negative, as instanced in the striking experiments described by Creed [1931].

We can now regard, not only the phenomena of simultaneous contrast, as embodied in Weber's Law applied to the visual phenomena of both brightness and colour, but also the phenomena of absent simultaneous contrast effects in ordinary perceptions of brightness and colour, as manifestations of the maintenance of constancy in activity within the visual spatial field. Successive contrast, on the other hand, can be

regarded as an indication of the tendency to constancy in the temporal field of visual activity. The word "contrast" is thus only a convenient expression without fundamental significance, and Weber's Law is only a partial representation of physiological facts, not valid at all under quite ordinary conditions.

It is evident that the theory now adopted is inconsistent with Young's three-colour theory of vision, as also adopted by Helmholtz and Maxwell. The phenomena of colour contrast, as observed in connection with coloured shadows and similar effects of both simultaneous and successive contrast, are left unexplained by this theory. We are thus driven towards Hering's conception of two pairs of primary colour sensations, besides a pair of sensations representing brightness and darkness, and always present in conjunction with colour sensation. What is simply white and black represents a balanced combination of the two primary pairs of colour sensations.

The theory stated above makes it possible to relate the maintenance of constancy in visual activity to various other respects in which an organism maintains constancy in its active relations with physiological environment—for instance the fundamental fact that throughout all the variations in intake and output of material and energy what is characteristic in structure and activities tends to be maintained in an adult organism. The manner in which, as Claude Bernard first pointed out, the composition and other conditions of the blood are kept constant, constitutes a special instance of this. A further instance, exemplified in much of my own work in physiology, is the manner in which the pressure of  $\text{CO}_2$  in the body, together with its hydrogen-ion pressure and oxygen pressure, tend to remain steady. In other words, the actual relations of environment to a normal living organism are such that what is characteristic of its life is maintained, though from the standpoint of mere physical science this cannot be expressed, since physical science is an abstract branch of knowledge which takes no account of what is fundamental in the phenomena of life, and thus, since we are living organisms, enters into all our perceived experience.

To realize the fundamental features in the physiology of vision, including colour vision, we must approach the subject as physiologists, and not merely as physicists or chemists, just as in the case of other physiological activities; and as I have tried to point out in more than one recent book, we get nowhere except into what seem to me the fogs of either vitalism or attempted mechanistic biology, if we treat an organism and its environment as separately existing things. What appears

light or dark, red or green, blue or yellow, depends, not on conditions which we can separately specify in either the environment or organism, but on relations throughout which we can trace the active maintenance of what is constant in visual activity. The field of vision is a field of co-ordinated activity, and not of mere receptivity.

It is evident that in the conclusions reached in the present paper, use has been made of biological, and not mere physical or chemical conceptions. The fact that in the sensory relations between organism and environment we can, as physiologists, discern one aspect of the maintenance of life as such, is part of biology, and not of abstract physical science.

It is well known that Goethe [1808] attacked ineffectively the teaching of Newton with regard to colour, and particularly the first proposition in Newton's *Opticks*, according to which "lights which differ in colour differ also in degrees of refrangibility." In the course of his voluminous writings on Colour, Goethe drew attention to the already well-known phenomena of coloured shadows, but unfortunately did not study them thoroughly enough. We can see from the present paper that criticism of Newton's statement is unanswerable. His statement is only true under definite physiological conditions, and lights of precisely the same refrangibility may have very different colours. But similar criticism can be directed generally against abstract physical reasoning which, following on the lines laid down by Galilei, disregards not only what is characteristic of biological experience, but also the fact that all elements in our experience have a definitely biological or psychological basis. The practical utility, for certain limited purposes, of making this abstraction is indubitable; but in biology we are, whether we are clearly aware of it or not, employing conceptions which can penetrate much more deeply.

#### SUMMARY.

1. Weber's Law, as applied to vision, only holds under exceptional conditions: we are ordinarily aware of arithmetical, and not geometrical, differences in either intensity or tint of illumination.

2. Coloured shadows and similar contrast phenomena do not represent what we are accustomed to call the natural tints of either of the two surfaces concerned, but exaggerated or distorted differences in these tints.

3. The phenomena represented in Weber's Law, in ordinary vision of differences in illumination and tint, and in colour contrast, are summed

up in the physiological conclusion that the field of visual activity, regarded as a whole, tends to exhibit constancy with respect to the prevalence within it of brightness and darkness, and of pairs of complementary colours, the latter being regarded as together equivalent to a degree of whiteness.

4. Young's three-colour theory of colour vision does not cover the phenomena of colour contrast.

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## THE EFFECT OF ŒSTRIN ON THE REACTIVITY AND SPONTANEOUS ACTIVITY OF THE RABBIT'S UTERUS.

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DURING pregnancy the uterus of the rabbit and of the human subject undergo specific variations in reactivity to certain substances and especially to the oxytocic factor of the posterior pituitary lobe [Robson, 1933 *a, b*]. These variations are, to some extent, dependent on the activity of the corpus luteum acting probably through a definite hormone [Robson and Illingworth, 1931; Robson, 1932 *a*; de Fremery, Luchs and Tausk, 1932]. It appears likely, however, that the changes which occur after the earliest stages of gestation are independent of the luteal activity, and the present investigation was performed to determine the effect of the ovarian hormone, œstrin, on the reactivity and activity of the uterine muscle.

### METHODS.

The experiments were performed on mature female rabbits weighing about 2 kg. With three exceptions the animals were ovariectomized on the day previous to the beginning of œstrin injections. In a number of cases a horn of the uterus was removed at the time of ovariectomy; part of it was examined histologically and the remainder used to determine the reactivity and activity of the muscle. The animals were again operated on after various periods of œstrin injections and the existing state of the uterus determined. Some of these animals were kept under observation for a further period and the condition of the uterus following cessation of œstrin administration determined.

In other cases no uterine tissue was removed at ovariectomy and the state of the uterus was determined only after œstrin injections and at subsequent periods.

In three further cases (Ra 351, 353, 357) the animals were operated on in the usual way and the condition of the uterus and ovaries noted, but

no tissue was removed. Oestrin injections were started as before, on the day following the operation, and the remainder of the experiment was performed as in the ovariectomized animals.

The uterine strips were suspended in oxygenated Ringer-Locke solution in 100 c.c. containers; the temperature (37° C.) and the oxygen supply were maintained constant. Records were taken on smoked drums. The degree of spontaneous rhythmic activity exhibited *in vitro* was determined in all cases, the strips of muscle being of similar length to that used in previous investigations. In the description the minus sign is used to represent slight or no spontaneous activity, while an increasing number of plus signs are used to denote various degrees of rhythmic contractions. The data are strictly comparable with those described for the pregnant animal [Robson, 1933 a].

The following preparations of oestrin were used in the experiments:

(1) An extract from pregnancy urine prepared as follows. Concentrated pregnancy urine was acidified with 10 p.c.  $\text{H}_2\text{SO}_4$  and extracted with ether continuously for 12 hours. The ether extract was extracted with sodium carbonate solution (which was discarded) and then with NaOH. The latter solution was acidified and extracted with ether; the substance left on evaporating the ether was dissolved in maize oil (=  $\alpha$  4 and  $\alpha$  5. I am indebted to Mr R. E. Illingworth for the supply of these preparations). Assay was by four injections spread over 36 hours.

(2) Progynon kindly supplied by Messrs Schering-Kahlbaum.

(3) A crystalline preparation of oestrin supplied by the British Drug Houses. This was prepared according to the method of Marrian and Hazelwood [1932]. The crystalline substance was dissolved in olive oil and assayed by four injections spread over 36 hours; a potency of 1000–2000 m.u./mg. was obtained, but the former figure has been used in the data given in this paper.

The preparation of the posterior pituitary lobe “pitocin,” kindly supplied by Parke, Davis and Co., was used in this investigation.

## RESULTS.

In estimating the reactivity of the uterine muscle to certain drugs an attempt was made in all cases to determine the minimal amount necessary to cause a motor effect. In the majority of cases this included a rise in the base line, but in some experiments small doses chiefly caused an increase in the height of the rhythmic contractions, and occasionally an increase in the rate of rhythmic contractions was the main effect. The







A



B



C

Fig. 1. To illustrate the progressive effect of cestrin injections on the uterus (Ra 384).

- A. Section of uterus before cestrin administration. Area=466. Reactivity  $>1.0$  unit.  $\times 15$ .
- B. Section of uterus after 10 days of cestrin administration. Area=1296. Reactivity 0.02 unit.  $\times 15$ .
- C. Section of uterus after 14 days of cestrin administration. Area=1800. Reactivity 0.005 unit.  $\times 15$ .

reactivity to oxytocin of the uterus before ovariectomy was, six out of seven animals investigated, low (see Table I) or very low; in one case (Ra 386) it was quite marked, 0.02 unit of pitocin (to 100 c.c. of solution) being sufficient to bring about a motor effect. After injection periods varying from 6 to 12 days during which doses ranging from 60 to 3200 m.u. of oestrin (in various forms) were administered, the reactivity to oxytocin was increased in every case but one (Ra 351). This animal received the smallest amount, namely 10 m.u. daily for 6 days. The increase in reactivity showed marked variations in the different animals and the degree of decrease in the minimal effective dose ranged from zero (Ra 351) to more than 200 times (Ra 384), and was quite marked in the majority of experiments. In one case (Ra 386) in which the initial reactivity was comparatively high, the oestrin injections did not greatly increase the reactivity to oxytocin. That the increase in reactivity can also be obtained in the presence of the ovaries is definitely shown by animal Ra 357 in which the injection of oestrin decreased the minimal effective dose of oxytocin from 0.1 to 0.004 unit.

In those experiments in which uterine tissue was not removed at the first operation the results subsequently obtained confirm the findings of the first series of experiments. For the average reactivity after oestrin injection was much higher than that shown by the first series of animals before any oestrin injections (Table I) and the minimal effective dose of oxytocin varied from 0.002 to 0.05 unit.

The data also definitely show that both time and dosage play an important part in the development of the reactivity to oxytocin, though marked individual variations among the animals were observed.

#### *Dosage.*

It appears likely that a certain minimal dosage of oestrin is necessary to obtain an effect on the uterine reactivity. Thus in Ra 351 six daily doses of 10 m.u. had no appreciable influence on the reactivity to oxytocin; that this may be an ineffective dose is also shown by animal Ra 353 in which the reactivity of the uterus actually decreased during a period when 10 m.u. of oestrin were being injected daily (10th to 14th days of the experiment). That this same dose may, on the other hand, be effective is shown by the results obtained in animal Ra 357 in which the reactivity increased by 25 times within 8 days. The relation of dosage to action on the uterine muscle is presented in Table II. When the reactivity previous to the injections is comparatively high even large doses of oestrin will not markedly decrease the minimal effective dose of oxytocin; thus the

reactivity in animal Ra 386 was only doubled as the result of the injection of 2700 M.U. of oestrin, the initial minimal dose of oxytocin being 0.02 unit.

TABLE II. Showing the relation between the period over which oestrin is injected, the dosage used and the increase in the uterine reactivity to oxytocin.

Animal Ra	Dose M.U.	Duration days	Reactivity before unit	Reactivity after unit	Increase in reactivity times
351	60	6	0.1	0.1	1
380	420	6	>0.6	0.1	> 6
357	70	7	0.1	0.004	25
384	450	10	>1.0	0.02	> 50
385	1900	10	0.6	0.006	100
386	2700	12	0.02	0.01	2
387	3200	12	>0.3	0.005	> 60
357	420	13	0.1	0.004	25
380	770	13	>0.6	0.01	> 60
384	800	14	>1.0	0.005	>200

#### *Time.*

Even with comparatively large doses of oestrin, however, the injections may have to be distributed over a fairly long period in order to obtain a high reactivity to oxytocin in the uterine muscle. Thus in animal Ra 380 the minimal effective dose of oxytocin after the injection of 420 M.U. of oestrin during 6 days was fairly high (0.1 unit), but an additional period of 7 days (during which 350 M.U. of the hormone were injected) further reduced the minimal effective dose by 10 times. In another experiment (Ra 382) 0.3 unit of oxytocin was necessary to cause contraction of the uterus after the injection of 390 M.U. of oestrin during 8 days, but the reactivity was decreased by 60 times after a further period of 7 days during which 310 M.U. of oestrin were injected.

After the cessation of oestrin injections the reactivity of the uterine muscle decreases, though the rate and extent of the decrease show marked variations in the different animals. The relation between time and decrease in reactivity are represented in Table III. It will be seen that in 10 cases out of 11 an increase in the minimal effective dose of oxytocin was noted. The exception is provided by animal Ra 357 in which, 7 days after cessation of oestrin injections, the reactivity to oxytocin was unchanged (0.02 unit). Animal Ra 376 shows a somewhat temporary exception though it received 50 M.U. of oestrin on the day after the first removal of uterine tissue; 9 days later the reactivity to oxytocin had actually increased by 4 times, but 25 days after the first uterine strip had been excised the minimal effective dose of oxytocin had actually increased by 15 times. The results do not suggest that there is a direct temporal

TABLE III. To show the fall in the uterine reactivity to oxytocin at various periods after cessation of oestrin injections. The value in column 4 is obtained by dividing column 3 by column 2.

Animal Ra	Reactivity before unit	Reactivity after unit	Decrease in reactivity times	Interval after cessation of injections days
387	0.005	0.01	2	5
379	0.02	0.02	1	7
374	0.02	0.08	4	7
386	0.01	0.03	3	8
378	0.05	> 0.3	> 6	8
377	0.01	0.1	10	10
385	0.006	0.3	50	13
383	0.01	0.1	10	13
381	0.02	0.1	5	16
380	0.01	0.02	2	17
376	0.02	0.3	15	25

relation between the time after cessation of oestrin injections and the fall in the reactivity to oxytocin, as comparatively small decreases were observed both at the earlier (*e.g.* Ra 387, 379, 376, 386) and later periods (*e.g.* Ra 380, 381). It must also be emphasized that even after a fairly long time had elapsed after the cessation of injections a comparatively high reactivity may be observed (*e.g.* Ra 330 in which, 17 days after the last oestrin injection, the minimal effective dose of oxytocin was still 0.02 unit).

#### *Spontaneous activity.*

The results obtained in those animals in which uterine strips were removed at the beginning of the experiments definitely show that injections of oestrin can increase the *in vitro* spontaneous rhythmic activity of the uterine muscle; this increase was observed in all animals investigated (Table I—Ra 351, 357, 380, 384, 385, 386, 387). Moreover, the increase in spontaneous activity becomes, as a rule, more marked as the period of injection is prolonged. At the same time, when the relation between spontaneous activity and reactivity is examined in those animals injected with oestrin (Table IV), there appears to be no connection between these values. It would thus seem that, although oestrin increases both the spontaneous rhythmic activity and the reactivity to oxytocin, the magnitudes of the increases in these values vary independently of one another.

After cessation of oestrin injections the spontaneous activity decreased in a majority of animals observed. Within the time limits investigated the fall in the rhythmic activity seems to be a slower process than the fall in reactivity to oxytocin; moreover, there appears to be little relation between the rate of decrease of these two values.

TABLE IV. To show the relation between the reactivity to oxytocin and the spontaneous activity in the uterus of animals injected with œstrin.

Animal Ra	Reactivity unit	Spontaneous activity
382	0.3	+
351	0.1	+
380	0.1	+
377	0.05	+
353	0.05	+
378	0.05	+
381	0.05	+
353	0.03	+
376	0.02	+
374	0.02	+
384	0.02	+
379	0.02	+
381	0.02	+
383	0.02	+
365	0.01	+
353	0.01	+
386	0.01	+
380	0.01	+
377	0.01	+
383	0.01	+
385	0.006	+
384	0.005	+
382	0.005	+
387	0.005	+
357	0.004	+
357	0.004	+
365	0.002	+

*Alterations in size.*

As is well known, œstrin produces a marked increase in the length and diameter of the uterus in addition to its actions on the reactivity and spontaneous activity of the uterine muscle; there also occurs proliferation of the endometrial glands which, however, remain straight and do not assume the appearance characteristic of the period of activity of the corpus luteum. An attempt was made to obtain a rough quantitative estimate of the effect of œstrin on the growth of the uterus by measuring the diameters of sections of pieces removed at various periods; in all cases diameters at right angles to one another were measured and multiplied by one another, the number obtained giving an index of the size of the uterus. It is fully realized that the results so obtained depend, to some extent, on the part of the organ from which the piece has been removed, on the state of contraction of the uterus at the time of removal, etc. At the same time the data are nevertheless significant in so far as large differences are concerned. They show that œstrin injections increase the size of the uterus and that the extent of this increase becomes more marked as the period of injections is lengthened (Table V), these effects

TABLE V. To show the relation of size of uterus to reactivity to oxytocin in animals before and after injection of oestrin and after cessation of hormone administration. (For details of injections see Table I.)

Animal Ra	Area of uterus	Re- activity unit	Time after ovari- ectomy days	Area of uterus	Re- activity unit	Time after ovari- ectomy days	Area of uterus	Reactivity unit	Time after ovari- ectomy days
384	466	>1.0	0	1296	0.02	11	1800	0.005	15
385	670	0.6	0	>2400	0.006	11	—	—	—
386	784	0.02	0	1600	0.01	13	—	—	—
387	999	>0.3	0	2912	0.005	7	—	—	—
379	3300	0.02	7	2500	0.02	14	—	—	—
380	2597	0.1	7	2308	0.01	14	—	—	—
381	2548	0.05	8	2400	0.02	16	—	—	—
382	1440	0.3	9	2544	0.005	16	—	—	—
383	1400	0.02	7	>2230	0.01	14	1560	0.1	27

being therefore similar to those obtained for the reactivity and spontaneous activity under similar conditions. In the injected animals, however, there appears to be no direct relation between the size of the uterus, its reactivity to oxytocin and the spontaneous activity exhibited by the isolated strips; and these results suggest that the extent of these increases in different animals varies widely and independently. After cessation of the injections the size of the uterus, like the reactivity and spontaneous activity, decreases.

#### DISCUSSION.

It has been shown that oestrin exerts three well-marked and concurrent actions on the uterus, namely (1) growth which involves both the endometrium and the muscle, (2) increase in the reactivity of the muscle to oxytocin *in vitro*, and (3) increase in the spontaneous rhythmic activity exhibited by the muscle *in vitro*. All these effects can be observed in the uterus of one and the same animal and are, moreover, progressive in character in so far as they become more pronounced as the period of treatment with oestrin is prolonged. It must be emphasized that these effects were obtained with various preparations of the hormone (including crystalline samples), though there are no data as to the relative activities of the various forms of oestrin.

The absence of any apparent relation in the injected animals between the reactivity to oxytocin, spontaneous rhythmic activity and size of the organ appears at first sight rather puzzling, in view of the fact that these three values are determined by the action of the same hormone. The same absence of any direct relation between the reactivity to oxytocin and the spontaneous activity was observed in the uterus of the rabbit and the

human subject during pregnancy [Robson, 1933 *a*, *b*]; here again the data showed that these values apparently varied independently. If all these effects are actually due to the direct action of the hormone on the uterus, then the reactivity of the organ for these various actions must be subject to wide variations. It would also seem to follow that the reactive systems affected by œstrin and responsible for reactivity to oxytocin, spontaneous activity and growth respectively cannot be identical.

With regard to the action of œstrin on the reactivity to oxytocin two factors appear to be of importance. In the first place a certain minimal dose of the hormone is necessary for the production of an increase in the reactivity; and secondly the action of the hormone must be distributed over a certain period and the reactivity may only increase gradually. This is the case even with fairly large doses as, for example, in Ra 382, where, after the injection of 390 M.U. over 8 days, the uterus only reacted to 0.3 unit of oxytocin while the additional injection of 310 M.U. over 7 days decreased the minimal effective dose of oxytocin to 0.005 unit. These results would suggest that the addition of œstrin *in vitro* cannot cause an increase in the reactivity to oxytocin (if it is assumed that the increases above described are actually due to direct action of the hormone on the uterus) owing to the necessarily limited period of action, and the recent results of Marrian and Newton [1932] show that such indeed is the case and that in very large doses œstrin *in vitro* actually decreases the reactivity of the uterine muscle to oxytocin.

The actual reactivity to oxytocin exhibited after the injection of œstrin is significant: in 12 out of 17 animals investigated the ultimate minimal effective dose of the posterior lobe hormone varied from 0.002 to 0.01 unit, while in four of the remaining five animals in which the reactivity was less marked the œstrin injections had been spread over a short period of 6 days, and it appears quite likely that the reactivity in these animals would have been increased if the injections had been continued for a longer period. Now the reactivity of the rabbit's uterus at or shortly after parturition varies from 0.01 to 0.0001 unit of oxytocin [Robson, 1933 *a*], figures which are very similar to those obtained after the injection of œstrin and which are higher than those observed under any other conditions. The similarity between these findings suggests that the œstrous hormone may be an important factor in the development of the uterine reactivity during the later stages of pregnancy and in the production of the high reactivity to oxytocin which characterizes parturition. Such a supposition is supported by the observation of Phillip [1929] that œstrin can be demonstrated in the blood of the rabbit during



the later stages of gestation. Also it is known that the action of oestrin in increasing the uterine reactivity cannot occur as long as the inhibitory hormone of the corpus luteum is being produced in any appreciable quantity, for oestrin cannot overcome the inhibitory effect of the luteal hormone [Robson and Illingworth, 1931; Illingworth and Robson, 1932], and furthermore the presence of oestrin is actually essential for the effect of the inhibitory luteal hormone itself upon the uterine muscle [Robson, 1932 *b*]. As the rabbit's uterus does not contract up to the 20th day of gestation with large doses of oxytocin (the exact time varies in different animals [Robson, 1933 *a*]), while vasopressin affects the muscle after the 16th day of gestation, it appears likely that the action of the inhibitory hormone ceases sometime between these two dates (*i.e.* between the 16th and 20th days), that the subsequent increase in the uterine reactivity to oxytocin is brought about by oestrin and that no appreciable quantities of inhibitory luteal hormone are then being produced.

In the human uterus the sequence of events appears to be similar. It is definitely established that the corpus luteum is not essential for the maintenance of gestation after the earliest stages [Ask-Upmark, 1926]. Moreover, the reactivity of the uterine muscle to vasopressin is already present at the 8th week of gestation although oxytocin does not, at that stage, exert any effect on the muscle [Robson, 1933 *b*]. These findings suggest that the action of the inhibitory luteal hormone ceases very early in pregnancy and that, as in the rabbit, the duration of its action may not be much longer than that of pseudo-pregnancy (if pseudo-pregnancy in the human subject be regarded as corresponding in length to the active period of the corpus luteum of menstruation). Subsequently the reactivity of the human uterus to oxytocin returns and gradually increases, reaching a maximum at parturition, and it seems quite possible that oestrin may play an important part in determining this series of events. It has indeed been established that the concentration of the hormone in the blood and its excretion in the urine gradually increases during pregnancy, reaching a maximum at parturition [Siebke, 1929; Zondek, 1930]. Moreover, the output of oestrin in the pregnant human subject is not interrupted by the removal of both ovaries at a very early stage of gestation [Waldstein, 1929]. These facts support the view that the reactivity changes in the human uterus during pregnancy are dependent on the action of oestrin. There are no data for the time relations of the action of oestrin upon the reactivity in the human subject, nor for the doses necessary for this action, but it appears at least possible (in view of the results obtained in

the rabbit) that the gradually increasing reactivity to oxytocin characteristic of pregnancy is, to some extent, dependent upon the continued action of the gradually increasing concentrations of œstrin present in the blood. The results also suggest that in the clinical use of œstrin, not only should large doses of the hormone be used, but the treatment should be continued over a fairly long period if an action on the uterine muscle is the aim in view.

The fall in the reactivity to oxytocin found in these experiments after the cessation of œstrin injections is interesting, as a similar phenomenon is observed in the uterus of the rabbit after parturition. In the latter case, indeed, the decline is very rapid and the minimal effective dose of oxytocin is always very greatly increased within 5 days of parturition; while after cessation of œstrin injections the reactivity to oxytocin decreases much more slowly. It seems possible that this difference may be due to the fact that in the injected animals the œstrin is not immediately excreted and that indeed some considerable time may elapse before the hormone is completely eliminated and its action on the uterus ceases. The fact that in one case (Ra 376) in which 50 M.U. of œstrin were injected on the day after the first operation which followed a 7-day period of hormone treatment, the reactivity actually increased after the cessation of œstrin injection and only decreased at a still later stage, supports the view that the action of œstrin on the uterus is continued for some time after the administration of the hormone has ceased. Even after cessation of hormonal action, however, the reactivity to oxytocin probably decreases slowly, and in the case above described (Ra 376) the uterus still contracted with 0.3 unit of oxytocin 24 days after the last injection of œstrin. A response to 1 unit of pituitrin has similarly been observed previously in an untreated animal 22 days after bilateral ovariectomy.

#### SUMMARY.

The injection of œstrin into ovariectomized and non-ovariectomized mature female rabbits causes (1) an increase in the reactivity of the uterus to oxytocin *in vitro*, (2) an increase in the spontaneous rhythmic activity exhibited *in vitro*, and (3) an increase in the size of the organ. There appears to be no correlation between the degree of these three effects.

The relation of dosage and of the period of injection and the effects of œstrin have been investigated. The progressive nature of the action of œstrin is stressed.

The state of the uterus after œstrin injections is compared with that obtaining at parturition and the possible rôles of the ovarian hormones in

determining the alterations in the uterine muscle of the rabbit and of the human subject during pregnancy and parturition are discussed.

Certain considerations bearing on the clinical use of œstrin are suggested.

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## A SIMPLE PHOTOELECTRIC COLORIMETER.

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ORDINARY colorimetric methods of measurement suffer from the disadvantage of requiring subjective judgments depending on the colour sensitivity of the eye of the observer. Their accuracy is limited by the eye fatigue which they engender and is dependent upon the quality of the light by which they are made. Most of them are also subject to other limitations: they require the use of complex and empirical calibration curves, are relatively slow, or necessitate fairly large quantities of material. The improvements brought about by the use of spectroscopic or photometric methods are still limited by the basic disadvantage of subjective measurement, intensities of two fields having to be balanced against each other or spectral bands located. This paper describes a simple colorimeter, using a differential copper copper-oxide photoelectric cell and two colour filters, which possesses none of these disadvantages. It is relatively cheap, simple, and quick to use, can be adapted to very small quantities of fluid, and has a linear calibration curve. It is especially well adapted to the measurement of the degree of oxygenation of hæmoglobin solutions, and is not subject to the usual disadvantages of the other optical methods (such as inaccuracies due to inactive hæmoglobin) which have been used for this purpose.

## PRINCIPLE OF THE METHOD.

The principle of the method can be seen from the diagram at the bottom of Fig. 1, which illustrates its applicability to the oxygenation reaction of hæmoglobin. Light from a suitable source passes through the absorption trough containing the solution being tested. Behind the trough two colour filters are fastened side by side in front of a differential photoelectric cell, so that part of the beam goes through each. The differential type of cell measures directly the difference in the amount of light falling on each half of its light-sensitive surface, and acts in effect

like two photoelectric cells working against each other. The two colour filters are so chosen as to match in a rough way the colours of the two end-points of the reaction being measured, so that when the colour goes from one extreme to the other there will be an increase in the amount of light going through one filter and a decrease in the amount going through the other. This reverses the distribution of light falling on the two sides of the cell, and with it the galvanometer deflection.

The working of the device is well illustrated by the hæmoglobin reaction for which it was specifically developed. As can be seen from the

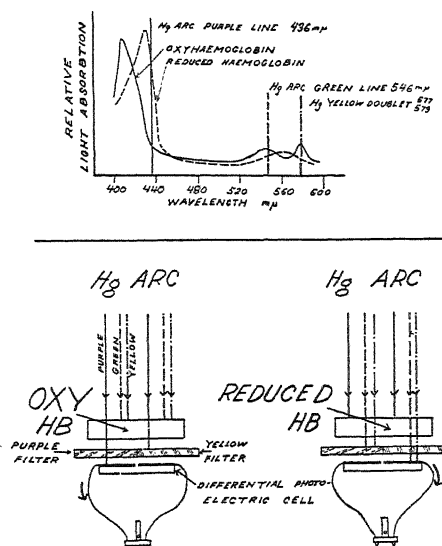


Fig. 1. Principle of photoelectric colour analyser applied to *Hb*.

absorption curves plotted at the top of Fig. 1, the mercury arc is a peculiarly well-adapted source for this colour change. Violet light of wavelength  $436\text{ m}\mu$ , where there is a very strong emission line is very much more strongly absorbed by reduced than by oxyhæmoglobin, while the yellow and green lines ( $579$ ,  $576$ , and  $546\text{ m}\mu$ ) are almost identical in wave-length with the peaks of the characteristic alpha and beta bands of oxyhæmoglobin, the position of which is used as the criterion for oxygenation by the Hartridge reversion spectroscope method. The Wratten D colour filter allows the violet light to pass almost undiminished and cuts out the yellow and green completely, while the yellow Wratten G filter allows these two colours to pass but is opaque to the violet. The

change from oxyhæmoglobin to reduced hæmoglobin in the absorption trough reverses the direction of the galvanometer current as shown in the diagram, partially oxygenated solutions producing intermediate deflections.

#### DESCRIPTION OF THE COLORIMETER.

A cross-section of the instrument is given in Fig. 2. It consists of light source, absorption trough, colour filters, photoelectric cell and galvanometer. The choice of source, trough, and galvanometer will depend upon the particular reaction being measured, the amount of material available, and the degree of sensitivity required. For most ordinary uses an incandescent lamp bulb, and a flat-sided trough about 1 cm. thick, with a low sensitivity galvanometer, or even a micro-ammeter, will prove

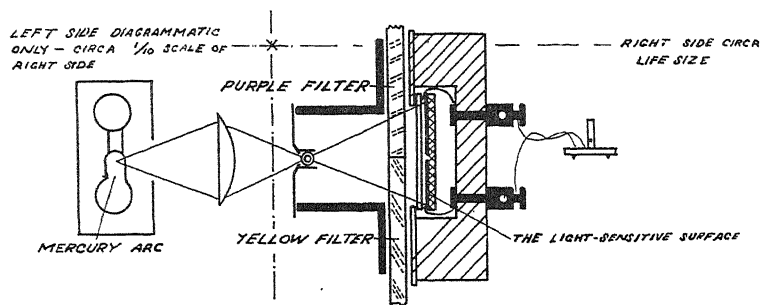


Fig. 2.

satisfactory. As illustrated the instrument is adapted to measuring the oxygen saturation of small quantities of hæmoglobin, flowing down a tube. A KBE 110-volt mercury arc lamp is used, focused on the absorption trough with a condensing lens. The trough itself is a 1 cm. length of 1 mm. glass capillary.

The photoelectric cell is the differential type of copper copper-oxide cell manufactured by the Cambridge Instrument Co. under Westinghouse patents. It consists of a copper disc about 2 cm. in diameter, with a layer of copper oxide on the front surface, and a thin metallic film on top of this. The surface is divided into two halves by a scratch along one diameter, and the terminals rest lightly on the metallic film, one on each half. This cell measures directly, and without driving potential, the difference in the amount of light striking the two halves. The colour filters are fastened to the photoelectric cell. They are adjustable from side to side, enabling the galvanometer to be brought to a convenient

balance during calibration, and are then clamped fast. The adjustment is so made that the two end-points give deflections which are approximately equidistant from the null point on either side. This use of a balanced system very greatly simplifies operation, and reduces to a minimum the ill effects of fluctuations in light source and lack of mechanical rigidity in the apparatus. It has proved of the utmost importance in getting reliable results.

With this apparatus, using the 1 mm. observation tube, and the mercury arc source, I have obtained galvanometer currents of about  $3 \times 10^{-7}$  amperes for the change from 0 to 100 p.c. oxygenation of a hæmolysed blood solution diluted 200 times.

#### CALIBRATION.

The galvanometer deflections corresponding to the two end-points are obtained directly either by successively running the fully oxygenated and the fully reduced hæmoglobin through the observation tube, or by using a three-compartment absorption trough, one compartment of which contains the unknown fluid, and the other two the end-point standards. The frequency with which these standards must be referred to depends upon the stability of the set-up, the steadiness of the lamp source, and the desired accuracy of reading.

The intermediate concentrations of the different pigments can be calculated by linear interpolation from the two end-points, when sufficiently dilute solutions, such as the method is well suited to, are used. The condition of linearity is that the amount of light absorbed by each substance be directly proportional to its concentration in the solution. In a parallel-sided trough, light absorption actually follows the well-known logarithmic expression:

$$\text{Amount of light absorbed} = \text{amount of incident light} \times (1 - e^{-k \times c \times d}),$$

where  $k$  is the specific light absorption of the substance,  $c$  its concentration, and  $d$  the thickness of the absorption trough. A simple calculation shows, however, that if a certain concentration absorbs 4 p.c. of the total light, then half that concentration will absorb 2.02 p.c. of the total light, which is only  $\frac{0.02}{2.02} = 1$  p.c. deviation from linearity. If an accuracy of 1 p.c. is desired, it is then necessary to see that the galvanometer deflection between end-points is less than 4 p.c. of the total deflection obtained by cutting off completely the light striking one side of the cell.

If this dilution condition has been complied with, the calibration curve is a straight line. To understand this, we need only make the

assumption, which has been often made before, and is in fact implicit in the calibration of the reversion spectroscope by the wedge trough method, that a partially oxygenated solution of hæmoglobin behaves optically like a mixture of the corresponding quantities of oxy- and reduced hæmoglobin. Let us, for the sake of simplicity, consider the case of a solution 50 p.c. saturated. The absorption curve of such a mixture will lie just half-way between that of the fully oxygenated and that of the fully reduced one given in Fig. 2, and in particular will cut the violet, green and yellow lines of the mercury arc spectrum just half-way between the intercepts of the two extreme curves. The violet light striking one side of the photoelectric cell will then be of an intensity mid-way between that of the two end-point solutions, and the same will be true of the yellow and green light striking the other side of the cell. Since the galvanometer records the algebraic sum of these different effects, its deflection will also be just half-way between its two calibrating end-point positions. Similarly, a solution one-third saturated will produce a galvanometer deflection one-third of the way from the fully reduced to the fully oxygenated positions, and so on.

The argument applies equally well to every reaction which involves any colour change, *i.e.* any alteration in the spectral absorption. In particular, it is applicable to mixtures of reduced hæmoglobin and carboxy-hæmoglobin; and at Dr Roughton's suggestion, I have used these mixtures to test empirically the linearity of the calibration curve, since known mixtures of reduced and carboxy-hæmoglobin are very much simpler to prepare than those of reduced and oxyhæmoglobin. The affinity of carbon monoxide for hæmoglobin is so great that one can make up a solution of saturated carboxyhæmoglobin, and subsequently remove practically all of the excess carbon monoxide without danger of appreciable dissociation. Such a solution was mixed in varying proportions with a solution of hæmoglobin previously reduced by bubbling nitrogen through it and adding a small quantity of sodium hydrosulphite, and its satura-

Actual p.c. COHb	Measured p.c. COHb photo-cell method	Maximum deviation
20	21	4
40	38	4
60	60	3
80	80	8

tion was then measured by the photoelectric cell method. The average of four determinations for each solution are given in the above table, as well as the maximum deviations of individual readings.



By this rough test the method appears accurate to about 4 p.c. for individual readings. The measurements were made under the very unfavourable conditions imposed by an absorption trough consisting of a cylindrical tube less than 2 mm. in internal diameter. This type of trough was dictated by the nature of the kinetic studies for which the method was being developed. With a larger observation vessel, and more light passing through it, a much higher degree of accuracy should be obtainable.

The linear character of the calibration curve is one of the principal advantages of the method. It reduces the labour of calculation and insures uniform accuracy throughout the whole colour range. It is, in this respect, more satisfactory for hæmoglobin reactions than the reversion spectroscopé, which has an empirical calibration curve, and which becomes unreliable at both high and low degrees of saturation.

#### SUMMARY.

A colorimeter using a differential copper copper-oxide photoelectric cell, and colour filters is described. Its advantages are:

- (1) Elimination of eye strain and subjective judgments.
- (2) Speed and ease of operation.
- (3) Linear calibration curve.
- (4) Availability for small quantities of material.
- (5) Simplicity and low cost.

It has been used to measure the degree of oxygen saturation of dilute hæmoglobin solutions.

THE KINETICS OF BLOOD PIGMENTS:  
HÆMOCYANIN AND HÆMOGLOBIN.

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## INTRODUCTION.

OXYGEN unites reversibly with the respiratory pigments, hæmocyanin and hæmoglobin. When the equilibrium state has been reached, as many molecules of oxygen are being bound per second by the pigment as are dissociating from it. This paper describes the individual measurement of these two opposing reactions observed in several kinds of hæmocyanin and hæmoglobin. It follows directly both in method and subject-matter the pioneer researches of Hartridge and Roughton [1923 *b*, 1925] on the kinetics of sheep hæmoglobin.

Measurements of the speed of the reactions of oxygen with the respiratory pigments can throw light directly on two groups of questions: (1) Is the speed with which oxygen is taken up or given off by the blood pigments slow enough to be a physiological limiting factor in the metabolism of the animal? (2) What is the mechanism by which the blood pigments bind oxygen? It is also hoped that these studies on the rates of reaction of the small oxygen molecules with large protein molecules may eventually throw some light on the mechanism of enzyme action, and serve as an introduction to a later kinetic study of catalytically active pigments.

## I. METHOD AND MATERIAL.

*The experimental method.*

The continuous flow method for following rapid reactions taking place in solutions, which was introduced by Hartridge and Roughton [1923 *a*], has been used. The two reacting fluids are driven at a high rate of speed into a common chamber where they are thoroughly mixed, and immediately passed down an observation tube, where the colour changes accompanying the reaction can be followed, each point along the

observation tube corresponding to a fixed calculable time after the initial mixing. The apparatus described by Hartridge and Roughton had two principal limitations which have been overcome in the present form. First, it was only adapted to relatively large amounts of material, which were not available when hæmocyanin or hæmoglobin from small animals was being studied. This has been remedied by constructing the device on a micro scale, the observation tube being a little less than 1 mm. in internal diameter. Second, the reversion spectroscope with which Hartridge and Roughton determined the degree of oxygenation of the hæmoglobin is not available for hæmocyanin, while even for hæmoglobin it can only give readings between about 25 and 75 p.c. saturation, thus missing the very important initial and end-points of the curve. Photoelectric cell methods have accordingly been developed, which are not subject to these weaknesses. The increase in total light absorption which accompanies the oxygenation of hæmocyanin when it goes from colourless to blue was used as the criterion for the extent of the reaction when this substance was being studied, while for hæmoglobin the change in relative absorption of different wave-lengths, which is indicated by the purple-to-scarlet shift, was measured. One such method is described in an adjoining paper [Millikan, 1933], and it is hoped in due course to discuss the remainder of the technique elsewhere.

In accordance with the procedure of Hartridge and Roughton the dissociation process has been measured by removing the oxygen liberated by the reaction as fast as it is formed by means of sodium hydrosulphite. This reduces the back reaction to zero. In measuring the rate of combination we have limited ourselves to the first portion of the reaction, where the backward component is negligible or can easily be allowed for. In the reduction process, a solution of oxygenated pigment is driven in one entrance of the mixing chamber and a solution of hydrosulphite in the other, the change of colour in the observation tube being then measured. It was necessary, however, to show that the oxygen absorber actually does act in this capacity, and does not attack directly the molecules of oxygenated pigment. By varying the initial hydrosulphite concentration, Hartridge and Roughton were able to show that in the reduction of hæmoglobin the reagent did act in this desired way, for, after a certain minimal concentration was reached, no further increase produced a change in the velocity of dissociation. I have been able to confirm this result for hæmoglobin, and to extend it to hæmocyanin. Furthermore, I have been able to show that the behaviour is completely different where hydrosulphite acts as a true reducer as in the reduction of methylene blue

to leuco methylene blue or of methæmoglobin to reduced hæmoglobin, and for these two substances the rate of reduction, as measured by the reciprocal of the time required to reach half completion, is directly proportional to the concentration of reducer, as one would expect from the simplest mass action considerations (see Table I, and Fig. 1).

TABLE I. Effect of hydrosulphite concentration on dissociation rate.

Substance and date	Time for half reduction (sec.)	
	0.2 p.c. $\text{Na}_2\text{S}_2\text{O}_4$	0.4 p.c. $\text{Na}_2\text{S}_2\text{O}_4$
Deoxygenation		
<i>Limulus</i> hæmocyanin, raw, Aug. 1931	$0.065 \pm 0.01$	$0.065 \pm 0.01$
<i>Limulus</i> hæmocyanin, dialysed, Aug. 1931	$0.075 \pm 0.01$	$0.075 \pm 0.01$
<i>Limulus</i> hæmocyanin, raw, Apr. 1932	$0.092 \pm 0.01$	$0.082 \pm 0.01$
<i>Maia</i> hæmocyanin, raw, Feb. 1931	$0.013 \pm 0.005$	$0.010 \pm 0.005$
Human hæmoglobin, July 1932	$0.017 \pm 0.002$	$0.014 \pm 0.002$
True reductions		
Methylene blue, Nov. 1930	$0.052 \pm 0.01$	$0.023 \pm 0.005$
Human methæmoglobin, July 1932	$0.053 \pm 0.012$	$0.025 \pm 0.010$

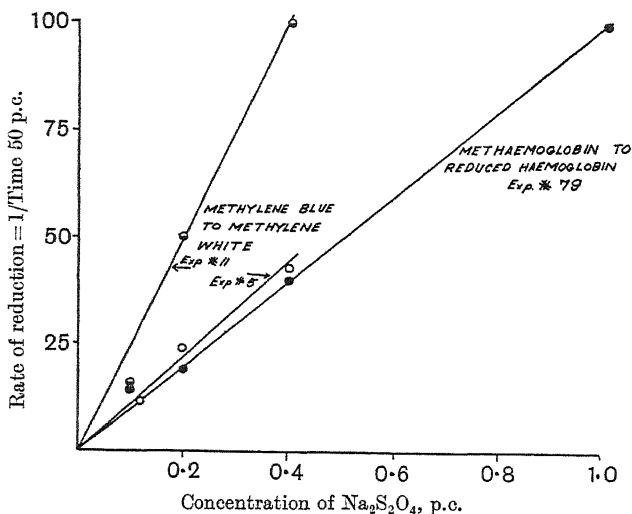


Fig. 1. Effect of reducer concentration in true reductions.

The control of the *pH* required special experimental precautions, because the sodium hydrosulphite used as an oxygen absorber acts as an acid in solution, and its oxidation products are more acid than it is. The reducing solutions of hydrosulphite were therefore made up in *M*/20 borate or phosphate buffers, with a small measured excess of  $\text{NaOH}$  added to bring back the reaction to that of the original buffer. Indicators could not be used to determine the *pH* with accuracy because of the bleaching effect of the hydrosulphite, so the reaction was in all cases determined with the glass electrode, and a large number of pre-

liminary tests were made to show that the final equilibrium  $pH$  was the same as that reached immediately after mixing in the observation tube. Since it was found that the effluent solutions became more acid on standing in the air, it was necessary to make the  $pH$  determinations during or immediately after a run.

In order to eliminate possible gradual changes in the pigments at different  $pH$ 's, it was the usual procedure to use as stock solutions in the apparatus a single batch of unbuffered pigment on the one hand, and on the other a series of buffered reducer solutions of different reactions.

#### *Substances used.*

Three principal respiratory pigments have been used in this study:

- (1) The hæmocyanin of the horseshoe crab, *Limulus polyphemus*, dialysed, and also in the native serum.
- (2) The serum of the spider crab, *Maia squinado*.
- (3) Laked sheep blood in dilute solution.

The first substance, in the purified form, is the best-established example of a "primitive" oxygen carrier, *i.e.* one for which the equilibrium dissociation curve is reproducibly a simple hyperbola. Its behaviour is further simplified by the fact that this curve is relatively insensitive to changes in hydrogen-ion concentration [Redfield, 1930]. *Maia* serum, on the other hand, represents a pigment which has "gone wild." Its equilibrium curve is of variable sigmoid form, and is enormously affected by  $pH$ . It has a very marked minimum affinity at  $pH$  6.2, and large increases on both acid and alkaline sides of this reaction [Hogben, 1926]. The native serum of the horseshoe crab has properties intermediate between these two extreme groups, the equilibrium curve being generally sigmoid in shape, and quite definitely affected in position by change of reaction, though not nearly as much so as that of the Crustacea. There is also a marked minimum affinity at a  $pH$  of 8.1 [Redfield, 1931].

Laked sheep blood was chosen for comparative study because it represents a hæmoglobin whose equilibrium curve has been very carefully worked out, and because it was the object of the earlier kinetic studies of Hartridge and Roughton with whose results the new data could therefore be compared. Some kinetic experiments have also been made on the hæmoglobins of man, frog, and pig, in order to compare the kinetic behaviour of a number of hæmoglobins with each other. The development of the micro method has for the first time made possible this type of investigation on the smaller or the more valuable animals (*viz.* frog, man), whose blood is only available in limited quantities.

*Limulus* serum, treated with toluol to prevent bacterial action, will keep many months without deterioration if stored at 0° C. I am extremely grateful to Dr A. C. Redfield for having generously provided me with a large amount of this material from Wood's Hole, Massachusetts. He drew the blood, filtered it through cheesecloth, added toluol, and shipped

it to me in the ice-boxes of fast liners. Handled in this way, the material remained in apparently good condition. Dialysis was carried out in the usual collodion sacks against distilled water at about 0° C., until the chloride content became less than 1 millimolar as judged by the amount of precipitate formed when an excess of silver nitrate was added to a nitric acid digest of the sample. Control tests were made with known salt solutions.

The formation of melanin proved extremely troublesome in the spider crab serum [Pinhey, 1930], *Limulus* blood being fortunately free of the tyrosinase which produces it. This tendency to blackening, the occasional presence of yellow lipochrome pigments, and the very great variation in the quality and concentration of pigment in spider crab blood made it a very much less satisfactory substance to work with than that of *Limulus* and reduced the accuracy of the results obtained with it. None of the devices used to combat these bad properties of the blood has been completely satisfactory.

Sheep hæmoglobin was prepared as described by Forbes and Roughton [1931] or Hartridge and Roughton [1923*b*], in order that the results might be comparable with those obtained in earlier work. Human hæmoglobin was obtained a few minutes before each experiment by defibrinating and laking with distilled water a few cubic centimetres of blood drawn from an arm vein. Two or three c.c. of red blood were sufficient for an entire kinetic run with thirty or forty individual points, but the poorer optical properties of hæmocyannin require for it greater concentration, so that 50–75 c.c. of the blue blood were usually necessary.

*Calculation of velocity and equilibrium constants.*

In calculating the velocity and equilibrium constants for "primitive" pigments, the following expressions were used:

$$(1) K = \text{equilibrium constant} = \frac{1}{p_{\text{O}_2} \text{ at 50 p.c. saturation}};$$

$$\text{Dimensions: } \frac{1}{\text{mm. Hg}}.$$

$$(2) K = \frac{k'}{k} = \frac{\text{association velocity constant}}{\text{dissociation velocity constant}}.$$

$$(3) k' = \frac{1}{a \times t_{50}} = \text{association velocity constant};$$

$a$  = initial concentration of both oxygen and reduced hæmocyannin in mm. Hg of O<sub>2</sub>;

$t_{50}$  = time of half completion of reaction;

$$\text{Dimensions: } \frac{1}{\text{mm. Hg} \times \text{sec.}}.$$

$$(4) k = \frac{0.7}{t_{50}} = \text{dissociation velocity constant};$$

$$\text{Dimensions: } \frac{1}{\text{sec.}}.$$

These expressions are a simpler and somewhat less accurate version of those given by Hartridge and Roughton [1925]. The following assumptions have been made in deriving them:

(a) The dissociation is monomolecular.

(b) In measuring the dissociation rate, the hydrosulphite absorbs the oxygen so quickly that there is no back reaction.

For these first two assumptions the evidence, both old and new, is now very strong. They should be valid quite independently of the validity of the remaining three.

(c) The association is bimolecular.

As far as the new kinetic studies go, the hyperbolic shape of the equilibrium dissociation curve is our only justification for making this assumption.

(d) In measuring the association rate, the back reaction is negligible.

(e) In measuring the association rate, the initial concentration of oxygen and reduced pigment are equivalent.

These last two assumptions can be checked in the following way. The complete differential equation for the association process is

$$\frac{dy}{dt} = k'(a - y)(b - y) - ky,$$

where

$y$  = concentration of oxygenated pigment,

$a$  = initial concentration of oxygen,

$b$  = initial concentration of reduced pigment.

This expression was given by Hartridge and Roughton. It can be integrated graphically upon substitution of any arbitrary values for  $k'$ ,  $k$ ,  $a$ , and  $b$ . If assumptions (d) and (e) are made this equation becomes

$$\frac{dy}{dt} = k'(a - y)^2,$$

which can very easily be integrated. Assumption (d) was checked by comparing the time of half completion of the reaction as calculated by graphical integration of these two equations, the substituted values being those of actual kinetic runs on *Limulus* hæmocyanin. The difference was just 10 p.c., which is less than the experimental uncertainty in the determination of this quantity. The final assumption (e) depends upon the experimental conditions. It was not always possible to make the concentration of the reactants exactly equivalent, but an excess of either one amounting to as much as 40 p.c. can be shown by integration of the corresponding equations to affect the half completion time of the reaction by less than 35 p.c., which is about the experimental uncertainty in this quantity.

The concentration units in which all of the constants are expressed is that in common use for equilibrium curve work, namely, "the equivalent partial pressure in millimetres of mercury." The reduced and oxygenated pigments as well as the dissolved oxygen can be very simply measured in this way, the pigment concentration being expressed in terms of its oxygen capacity. Roughton [1932] has given the thermodynamic

justification for the unit. It is used here because of its convenience, but it can readily be converted into the usual chemical notation by means of the Bunsen solubility coefficient and the gas constant.

It has been pointed out by Adair and by Hartridge and Roughton that the same kinetic and equilibrium equations are obtained if the pigment molecule contains any number of places for oxygen instead of only one, provided that these valencies are all equally strong and are independent of each other. For purposes of calculation one "molecule" of the pigment would, however, be defined as the amount of substance combining with one molecule of oxygen.

## II. EXPERIMENTAL RESULTS.

### *Under physiological conditions.*

The direct physiological interest in kinetic experiments on the blood pigments lies in the answer to the question: are these reactions slow enough to be limiting factors in the metabolism of the animal? In the vertebrates the question is complicated by the fact that here the pigment is carried in corpuscles, diffusion and chemical reaction velocity becoming joint factors in determining the total rate of gas exchange, as has been shown by Roughton [1932]. Hæmocyanin, however, is dissolved directly in the blood, and this complication does not exist in its case. *In vitro* experiments on hæmocyanin therefore give us a much more direct picture of what goes on in the organism than corresponding ones on hæmoglobin. In two other respects the experimental conditions in the kinetic studies of the blue bloods approach more closely those obtaining in the living animal. Due to optical properties of the pigments, the concentration of hæmoglobin must be reduced to one-hundredth or less of that in the corpuscles in order to make kinetic measurements, and it is conceivable that this great dilution may considerably affect the measured velocity constant of the reaction. Hæmocyanin, however, can be studied at practically blood concentrations. In the second place, normal room temperature, at which these experiments can most easily be carried out, is a normal one for the blue-blooded animals, but is below the body temperature of the mammals.

Table II summarizes the results of kinetic dissociation runs made on the native sera of the spider crab, *Maia*, and the king crab, *Limulus*, at room temperature and at hydrogen-ion concentrations in the neighbourhood of the physiological range [Quagliariello, 1916]. In Fig. 2 are plotted the relevant points of two of the individual experiments. Several



TABLE II. Rate of dissociation of arthropod sera under physiological conditions.

Exp.	Species and date	pH	Time, 50 p.c. reduction sec.
50	<i>Maia</i> , Nov. 1931	7.4	$0.017 \pm 0.005$
51	„ Nov. 1931	7.5	$0.020 \pm 0.008$
		(interpolated)	
52	„ Nov. 1931	7.5	$0.015 \pm 0.008$
		(interpolated)	
71	„ June 1932	7.5	$0.020 \pm 0.004$
	Average, <i>Maia</i> , c. 20° C.		$0.017 \pm 0.006$
46	<i>Limulus</i> , Aug. 1931	?	$0.065 \pm 0.020$
56	„ Feb. 1932	9.0	$0.075 \pm 0.010$
57	„ March 1932	8.0	$0.075 \pm 0.010$
59	„ April 1932	8.0	$0.075 \pm 0.010$
60	„ April 1932	8.0	$0.080 \pm 0.015$
	Average, <i>Limulus</i> , c. 20° C.		$0.075 \pm 0.010$

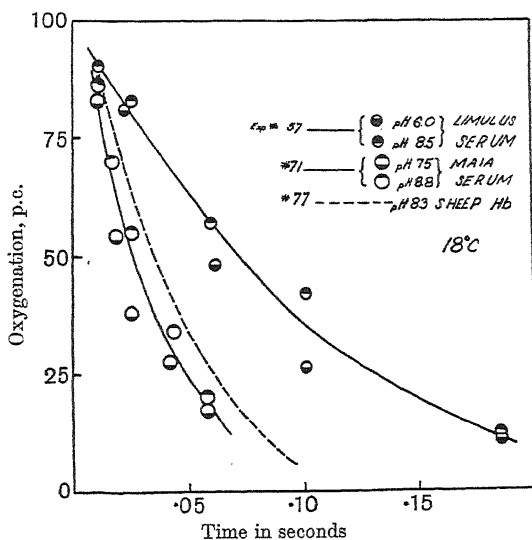


Fig. 2. Rate of dissociation of sera; physiological conditions.

of the points are averages of two or more readings to avoid congestion in plotting. A corresponding curve for sheep hæmoglobin is included for reference. It will be seen that the rate at which oxyhæmocyanin dissociates is of the same general order as that of oxyhæmoglobin, the half reaction taking from a sixtieth to a tenth of a second. There is, however, a very considerable difference between the two hæmocyanin species, the relatively active spider crab possessing blood which yields up its bound oxygen much more rapidly than the more sluggish horseshoe crab. It is improbable that this difference is of any physiological significance, since

even the most slowly dissociating pigment gives up half its oxygen in one-tenth of a second, whereas the time of passage of blood through the tissue capillaries is never less than several seconds.

The rate of oxygen uptake at physiological concentrations is a much quicker affair than the reverse process, reaching half completion in less than 1/300 second for both *Limulus* and *Maia* serum, which is very much too fast to make it a limiting factor in the metabolism of the animal.

The observation tube of the kinetic apparatus does not give a strictly accurate picture of what takes place in the gill capillary, since in the former the oxygen is used up from the supply initially sent down the tube with the reduced pigment, and there is therefore a progressively lower oxygen tension as we proceed along the tube; in the intact animal, on the other hand, oxygen is drawn from outside the capillary, where it exists at a fairly constant concentration. In the kinetic experiments, the initial oxygen tension of about 300 mm. of Hg was chosen so that the average oxygen pressure as the fluid went down the tube would be about that of aerated water, but it is not claimed that the value of 1/300 sec. for half completion tells anything more than the order of magnitude of the rate in the capillary. This happens to be all that is needed to answer the physiological question of limiting factors in oxygen metabolism.

*The comparative speeds of dissociation of different blood pigments.*

The hæmoglobins of three mammals and one amphibian have been studied under identical (though not physiological) conditions, as well as the hæmocyanins of *Limulus* and *Maia*. The results are summarized in Table III. For these hæmoglobin experiments the blood was hæmolyzed

TABLE III. Rates of dissociation compared with oxygen affinity. pH 8.6, 22° C.  
(Kinetic values all obtained in same experimental run.)

Hæmoglobins	Reduction time $t_{50}$	Reciprocal affinity $p_{O_2} - 50$ p.c. sat. (mm.)	Authority for affinity values
Frog	0.020	18	Macela and Seliškar [1925]
Sheep	0.028	3	{ Forbes and Roughton [1931] Hartridge and Roughton [1923b]
Man	0.038	1	Macela and Seliškar [1925]
Pig	0.047	Unknown, but low	
Hæmocyanins			
Spider crab, <i>Maia</i>	0.025	16	Hogben [1926]
King crab, <i>Limulus</i> :			
Dialysed	0.080	3	Redfield [1930]
Native	0.075	17	Redfield [1930]

and diluted two hundred times in borate buffer. No attempt was made to remove the stromata, salts, or plasma proteins, but their concentration would be low in the final dilute solution. In any case, the effect of salt concentration on the rate of dissociation is probably not large, as its

removal is seen to have little effect on this quantity in *Limulus* hæmocyanin (Table III). The value for *Maia* hæmocyanin given in the table is for the native serum, as no extensive runs were made on this material after dialysis. In Table III are also given the partial pressures of oxygen in equilibrium with half-saturated pigment solutions, as a rough measure of the oxygen affinity. Their significance will be discussed in a later section.

*The order of the dissociation reaction.*

The kinetic dissociation curve of both hæmoglobin and hæmocyanin has been found to be monomolecular within the limits of error of the

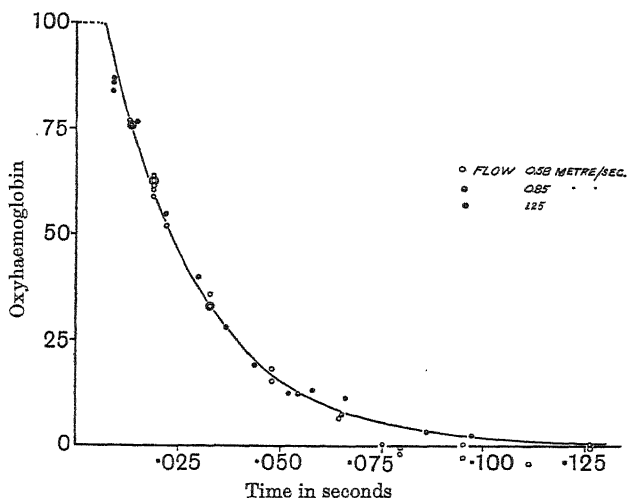


Fig. 3. The dissociation of sheep hæmoglobin. Exp. 77, pH 8.3. Curve constructed from equation  $y/100 = e^{-44.5(t - 0.0074)}$ ; reduction velocity constant =  $44.5 \frac{1}{\text{sec.}}$ ; time of induction = 0.0074 sec.

experiments, quite independent of the shape of the equilibrium dissociation curve. The data of one experiment on sheep hæmoglobin are plotted in Fig. 3, and replotted logarithmically in Fig. 4. These findings completely confirm the earlier results of Hartridge and Roughton obtained with the reversion spectroscope on the same material, and they furthermore extend the measurements to much higher and lower saturation ranges where the test for monomolecularity becomes more severe. The standard deviation from the logarithmic curve of all points above 3 p.c. saturation is 3.3 p.c. (This is raised to about 5 p.c. if we include the points on the extreme tail of the curve, which were thrown somewhat low by a systematic error of manipulation.) The mutual consistency of points

made with widely differing rates of flow gives satisfactory evidence that these results have a real meaning and are not experimental artefacts.

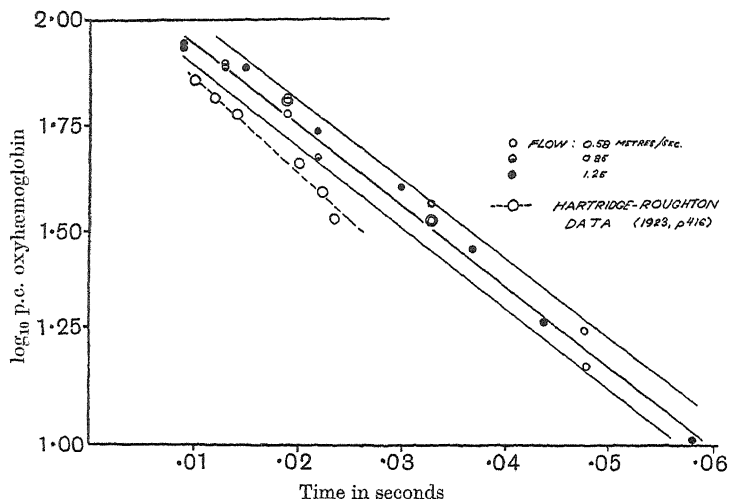


Fig. 4. The order of the dissociation of sheep hæmoglobin. Exp. 77, pH 8.3.

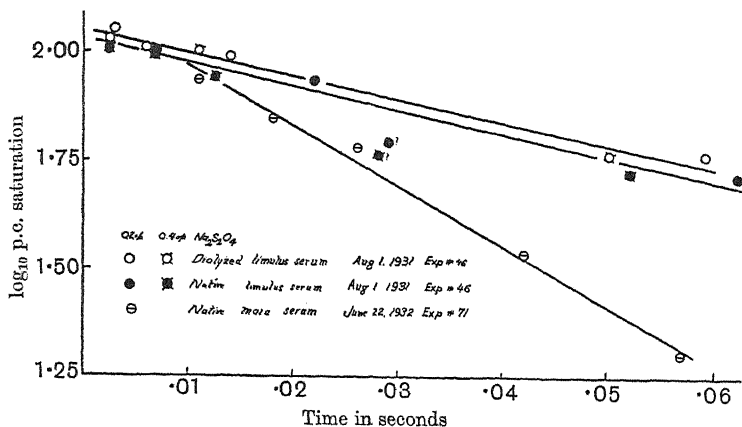


Fig. 5. The order of dissociation of hæmocyanin.

Much less accuracy is obtainable with hæmocyanin, but within the wider limits of experimental error, both *Maia* and *Limulus* sera show the same monomolecular dissociation curves (see Fig. 5), the logarithm of the amount of oxyhæmocyanin decreasing uniformly with increasing time. This is true for *Limulus* serum both before and after dialysis, though the

equilibrium curve is much affected in shape and position by this purification. Neither the shape nor the position of the kinetic dissociation curves is affected by a two- or threefold change in the concentration of hæmocyenin (*Limulus*).

*Effect of pH on dissociation rate.*

The effect of varying the pH on the rate of oxygen dissociation differs very considerably from substance to substance. The shifts will be most easily understood in relation to the corresponding changes in equilibrium behaviour, for which the relevant data have been summarized in Fig. 6.

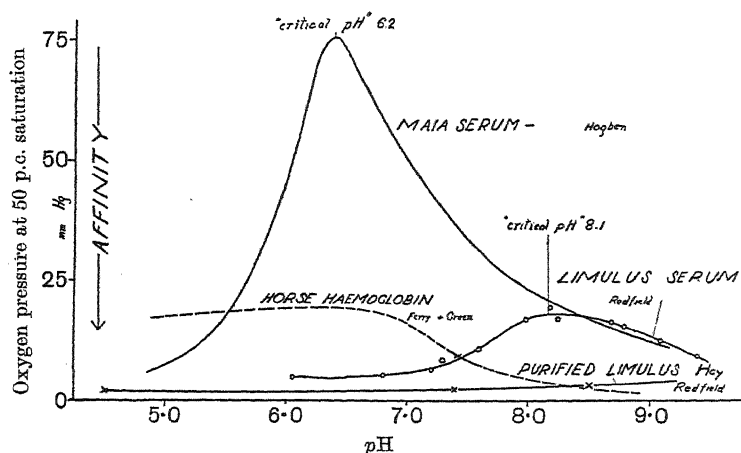


Fig. 6. pH effect on equilibrium curves: hæmocyenin and hæmoglobin.

It had been previously found by Hartridge and Roughton that the dissociation rate of dilute sheep hæmoglobin was much higher in acid than in alkaline solution. By repeating their experiments on the new apparatus, I have been able fully to confirm their results, and to show that they can also be carried over to human material (Fig. 7). As was expected from the greater affinity of human hæmoglobin for oxygen [Forbes and Roughton, 1931], the rate of dissociation was in every case considerably lower than that of the sheep, but the character of the change with pH was found to be the same. If the velocity constant is plotted against pH, the curve is seen to resemble the titration curve of a monovalent acid with a  $pK$  of about 7, there being a low constant rate at the alkaline end, a high constant rate at the acid end, and an intermediate transition portion.

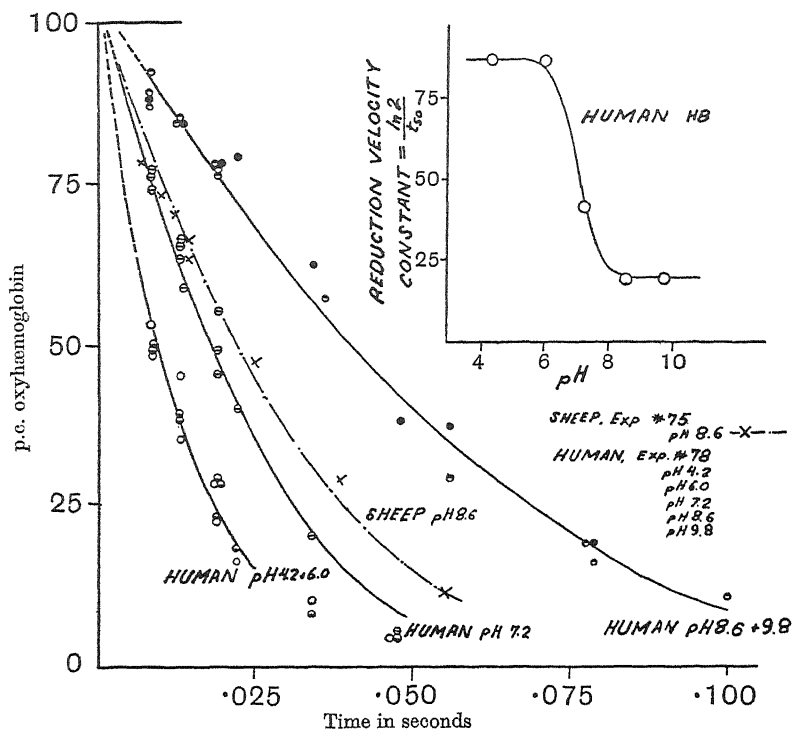
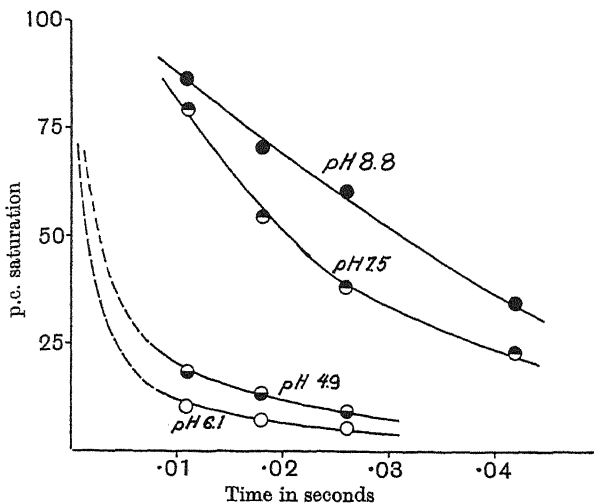


Fig. 7. pH effect on rate of dissociation: human haemoglobin.

Fig. 8. pH effect on rate of dissociation: *Maia* serum, 19°C. Exp. 71. Native serum.

The hæmocyanins of *Limulus* and *Maia* show completely different effects, *Maia* being very sensitive indeed and *Limulus* relatively insensitive (Figs. 8, 9). This reflects the general equilibrium behaviour plotted in Fig. 6. It will be seen that the rate of dissociation of *Maia* serum increases enormously as the solution becomes more acid, but there is no marked slowing up or "come back" in the rate after the critical pH (6.2) has been passed, although the oxygen affinity does show such a marked return. *Limulus* serum, on the other hand, shows only a small pH effect, the rate increasing slightly with acidification. Here also there

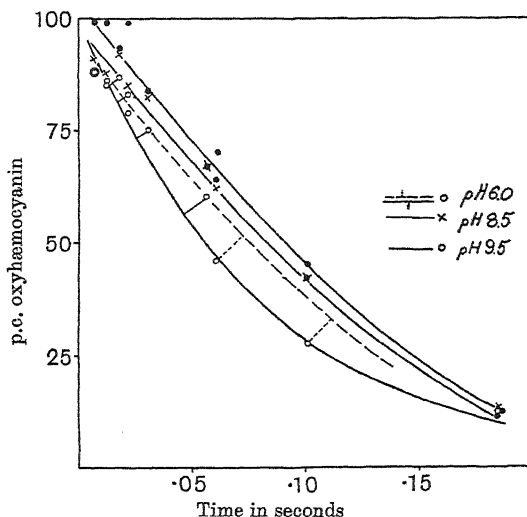


Fig. 9. pH effect on rate of dissociation: *Limulus* serum.  
Exp. 57. Points are individual readings.

are no signs of a return to lower speeds on the acid side of the "critical pH" (8.1). The pH effect on this species is, however, so small as to be within the rather wide limits of experimental uncertainty. No satisfactory experiments on the pH effect of the dialysed *Limulus* material have been made.

#### *Effect of temperature on the dissociation rate.*

Increasing the temperature greatly speeds up the dissociation rate of purified *Limulus* hæmocyanin, just as Hartridge and Roughton found it to do for sheep hæmoglobin. The experimental uncertainties in the hæmocyanin temperature work were, however, too great to allow an exact calculation of the temperature coefficient. The value of  $Q_{10}$  is probably

about 3.8 (the same as the hæmoglobin value), though one experiment gave as low as 2.2. The shift in the equilibrium constant for this material has been found by Brown [1933] to have a  $Q_{10}$  of 2.3.

*The rate of combination of oxygen with reduced pigment.*

The new experiments have not yielded quite as accurate results for the speed of oxygen association of hæmocyanin as Hartridge and Roughton were able to obtain for sheep hæmoglobin, the values given in Table IV

TABLE IV. Rate of combination of oxygen and reduced pigment.

Exp.	Material	Initial average conc. of reactants mm. Hg of O <sub>2</sub>	Time of half completion sec.	Association velocity constant assuming bi- molecularity
		<i>a</i>	<i>t</i> <sub>50</sub>	<i>k'</i>
67	<i>Limulus</i> hæmocyanin, dialysed	30	0.012	2.8
68	" "	32	0.008	3.9
65	Sheep hæmoglobin, diluted 1 : 200	25	0.010	4.0
	Sheep hæmoglobin	—	—	4.5-6.3
	Hartridge and Roughton [1925, p. 674], converted to these units			

being possibly in error by as much as 40 p.c. The points of the individual curves are too scattered to give definite information about the order of the reaction. Bimolecularity is assumed in calculating the velocity constant of dialysed *Limulus* serum simply because of the shape of the equilibrium curve. It is also assumed in deriving the same constant for sheep hæmoglobin, in order to compare the results with those of Hartridge and Roughton [1925]. It will be seen that with equivalent initial concentrations of the reactants the two pigments react with oxygen at roughly the same rate of speed.

In order to catch the association reaction at all, a redesigned form of the kinetic apparatus was required, which would allow greater dilutions to be used. This apparatus has an observation tube 2 mm. in diameter (instead of the usual 1 mm. tube), and a 4-jet mixer (instead of the usual 2-jet type). No other changes were made in the experimental arrangements.

### III. DISCUSSION OF RESULTS.

*The relation of "on," "off," and equilibrium constants.*

The purified hæmocyanins of *Limulus*, *Helix*, and *Busycon* are the only respiratory pigments now known which reproducibly give hyperbolic equilibrium curves and for which, therefore, the very simple Hufner assumptions can reasonably be made. One of these substances is the



logical choice for a comparison of the observed equilibrium affinity with that calculated from the measured rates of the two opposing kinetic reactions. These values for dialysed *Limulus* hæmocyanin are given in Table V.

Association		Dissociation		Equilibrium	
Exp.	$k'$	Exp.	$k$	Calc.	Observed [Redfield]
67	2.8	69	7.4	0.38	0.40
68	3.9	—	—	0.53	—

Exps. 67, 68, and 69 were all made on the same material and on adjacent days. The "observed" value of 0.40 for the equilibrium constant is interpolated from the data of Redfield [1930], and Brown [1933]. I have been able to confirm both the shape of the curve and the value for the equilibrium constant by these workers, on material identical with that used in the kinetic measurements, though the uncertainty of my  $K$  value was considerably greater than theirs, being about 0.1. The possible errors for  $k'$ ,  $k$ , and  $K$  as judged from the experimental uncertainties are 20, 50, and 25 p.c. respectively. The agreement between observed and calculated values of the equilibrium constant is therefore as good as could have been expected, being within 5 p.c. in one case and 35 p.c. in the other.

The situation in regard to hæmoglobin is much less satisfactory. Hartridge and Roughton [1925] obtained about the same agreement between the velocity and equilibrium constants on sheep hæmoglobin as have now been obtained on *Limulus* hæmocyanin. They had determined equilibrium curves on this material, using the reversion spectroscope, and had found them to be hyperbolic in shape. Later investigation, however, especially by Forbes and Roughton [1931], has reproducibly given sigmoid curves for dilute solutions of sheep hæmoglobin. The simple Hufner theory is no longer able to provide an accurate description of these experimental facts, and we must seek agreement between kinetic and equilibrium data on the basis of other primary assumptions.

Kinetic data can be of help in assigning relative merit to other theories of the nature of the oxygen-respiratory pigment complex, although the quantity and accuracy of the kinetic results so far obtained are not sufficient to give a definitive award to any one. The Hill theory, which has generally been regarded as untenable in its original form since the two independent determinations of Adair and Svedberg, which gave agreeing values for the molecular weight very different from that predicted by this hypothesis, also fails in its prediction of kinetic behaviour of sheep

hæmoglobin in dilute solution. The kinetic equations given under "Method" for a simple pigment in which  $n = 1$ , can very easily be generalized for any integral values of  $n$ , and the velocity constants can be obtained in the same way. The very careful equilibrium curves of Forbes and Roughton, made under conditions which were duplicated as closely as possible in the present kinetic studies, have inflections which give  $n$  values between 2 and 3. The equilibrium  $K$  calculated from  $k'$  and  $k$  differs, however, from that obtained directly from the equilibrium curve, being five times too small for  $n = 2$  and twenty times too small for  $n = 3$ . The combined possible errors, though fairly large, are very much less than this discrepancy. Since all values of  $n$  greater than 1 give completely unacceptable calculated values for the equilibrium constant, a theory based on mixtures of two kinds of such molecules (in which, for example, one is of the  $n = 2$  form, and the other of the  $n = 4$  form) such as Redfield has used in interpreting equilibrium curves, would have great difficulty in explaining the results of the kinetic measurements.

There remains the intermediate compound hypothesis suggested by Adair. This theory adequately accounts for the shapes of the carefully made equilibrium curves of Adair [1925], Ferry and Green [1929], and Forbes and Roughton [1931], if the four arbitrary constants predicted by it are suitably chosen. Its principal disadvantage, as a theory, is that it allows too much latitude in the choice of the constants. This lack of specificity is so great that until more evidence is available on the probable values of some of these constants, the attempt to correlate the kinetic and equilibrium data is perhaps futile. At this stage, however, two considerations do deserve emphasis:

(a) Analysis shows that it is quite possible for the intermediate compound hypothesis to lead to an accurately monomolecular curve for the dissociation velocity, even if the equilibrium curve is sigmoid (*e.g.*  $n = 2$  to 3 on Hill's notation). It is hoped later to develop this matter more fully.

(b) Preliminary experiments by Roughton have shown that, under some conditions, there may be a difference between the "slow equilibrium" finally reached when oxygen and hæmoglobin are shaken together in tonometers, and the "quick equilibrium" reached in a fraction of a second when oxygen solutions and hæmoglobin solutions are mixed together in the rapid reaction apparatus. The photoelectric methods are particularly suitable for following up this point further. If it were indeed established it would obviously be of prime importance in the correlation of kinetic and equilibrium data.

*The molecular weights of blood pigments.*

We have seen how similar in their kinetic properties are hæmoglobin and hæmocyanin, and the equilibrium work of the last few years has but emphasized the essential likeness of these two groups of substances in their behaviour towards oxygen. The differences between the two groups are in general no greater than interspecies differences within each group. It is appropriate, therefore, to ask of what significance is a thirty- to seventyfold difference in their molecular weights, Svedberg's ultracentrifugal determinations having given a value of 5,000,000 for the weight of *Helix* hæmocyanin and 2,000,000 for *Limulus* hæmocyanin as compared with 68,000 for mammalian hæmoglobins. Adair's intermediate compound hypothesis is as easily capable of explaining sigmoid dissociation curves with  $n = 30$  or  $n = 70$  as with  $n = 4$ . This theory further suggests a very simple qualitative explanation for the observed change in shape of the curves under different conditions of salt concentration, for if the relative dependence of one oxygen valence on another is determined by the salt concentration, such changes are predicted. For example, in the case of *Limulus* hæmocyanin, the hyperbolic curve obtained for the purified material is predicted by the intermediate compound hypothesis if the tightness with which one oxygen molecule is bound in no way affects the equal tightness with which its neighbour is bound. This will be so no matter what is the molecular weight, and will also explain the indifference of the hyperbolic equilibrium curve of purified *Helix* hæmocyanin to its molecular weight, the Stedmans having shown that the position and shape of the curve are practically independent of the reaction between pH 4.0 and 9.0, whereas Svedberg has found the "5,000,000" molecules to be stable only between pH 4.5 and 7.5, beyond which they break up into smaller fragments. The addition of salts must now be supposed to introduce restricting influences on the oxygen valencies, so that they become to a certain extent dependent on one another. The altered equilibrium constants when substituted into the intermediate compound equation for oxygen equilibrium will now give it an S-shape, such as that observed experimentally.

Although Adair's theory was put forward on the basis of definite molecular weight data, it is now seen that its usefulness persists even in the absence of the support furnished by the molecular weight, and molecular size becomes of relatively little importance in fitting the oxygen data, so long as one molecule has at least several places for oxygen. It is quite possible that the giant hæmocyanin molecule is made up of

hæmoglobin-like "sub-molecules," in each of which  $n = 4$  or some other small number, but so far we have no experimental evidence either for or against this hypothesis.

*The equilibrium condition.*

When a solution of hæmocyanin or hæmoglobin is in equilibrium with a given partial pressure of oxygen, just as many molecules of oxygen are being bound by the pigment every second as are dissociating from it. If any change in conditions produces an increase in the equilibrium affinity, this can only mean that there has been an increase in the rate at which oxygen molecules have been joining on or a decrease in the rate with which they have been coming off, or both. This is true quite independently of any theory of the mechanism by which they are bound. Hartridge and Roughton found that for sheep hæmoglobin it was the dissociation reaction which bore the brunt of such a change, the joining on process being little if at all affected by changes in  $pH$ , salts, or temperature. The experiments on hæmoglobins of several animals described in Section II show that qualitatively at least this principle may be extended to interspecies differences, the higher the affinity, the slower being the dissociation (Table III). There are difficulties, however, when we attempt to apply the same principle to the two hæmocyanins which have been investigated. The native hæmocyanins of *Maria* and *Limulus* at  $pH$  8.6 have approximately equivalent affinities, but their dissociation rates differ by a factor of three. Furthermore, the large increase in the affinity of *Limulus* serum for oxygen brought about by dialysis fails to be reflected in any considerable change in the rate of dissociation. Another exception is found in the  $pH$  behaviour of both types of blue blood, for on the acid side of the "critical  $pH$ " acidification brings increased affinity for oxygen, but fails to bring marked slowing down of the dissociation process.

Two possible explanations for these exceptions suggest themselves:

(1) there is some compensating change in the rate of the combining reaction, or

(2) the published equilibrium curves fail to portray the equilibrium conditions at the moment of the kinetic measurements.

More extensive kinetic association data or more information about "fast" and "slow" equilibrium curves will be needed for a decision between the two.

*Comparative properties of hæmoglobin and hæmocyanin.*

A number of outstanding properties of hæmoglobin and hæmocyanin have been listed in Table VI. A glance at this table will show how our newer kinetic evidence has reinforced the results of equilibrium work in bringing out the essential similarity in the oxygen-binding behaviour of

TABLE VI. Comparative properties of hæmoglobin and hæmocyanin.

Properties of equilibrium curve	Hæmoglobin	Hæmocyanin
1. Shape, native state	Always sigmoid	Always sigmoid
2. Shape, purified	Probably always sigmoid	Sometimes hyperbolic: often sigmoid
3. pH effect, native state	None ( <i>Urechis</i> ) Moderate (mammals) Large (fish)	None (pure <i>Helix</i> ) Moderate ( <i>Limulus</i> ) Large ( <i>Maia</i> )
Kinetic properties		
4. Approximate time of dissociation, $t_{50}$ , 20° C., pH <i>circa</i> 8.6	1/40–1/20 sec.	1/40–1/10 sec.
5. Character of dissociation	Accurately monomolecular	Monomolecular within experimental limits
6. Effect of decreasing pH on rate of dissociation	Increase	Increase or small change
7. Effect of increasing temperature on rate of dissociation	Great increase $Q_{10}=3.8$	Probably great increase $Q_{10}=2.2?–3.8$
8. Approximate rate of combination, $k'$ (assuming bimolecularity)	4.0 (new)–6.3 (H.R. 1925)	2.8–3.9
Other properties		
9. Metal: Element	Iron	Copper
Pyrrol prosthetic group	Yes	No
10. Ratio, metal: oxygen molecules	1 : 1	2 : 1
11. "Met" oxidation product	Yes	Yes (Conant)
12. Addition products beside oxygen: CO	Yes	?
KCN	Probably no	Yes
13. Molecular weight	68,000 (mammals) millions ( <i>Arenicola</i> )	2,000,000–5,000,000 for 2 species (no Crustacea)

these pigments, and we have seen above that the molecular weight differences need not affect this similarity. Of what significance are the other differences, such as the metallic element, the ratio of metal to oxygen bound, the existence of other loose complexes? The answer to this question is probably closely bound up with the catalytic rôle of iron and copper compounds in biological processes, and it is from the further study of oxygenation and oxidation, of oxygen transport and oxygen catalysis—perhaps by kinetic methods—that we may hope for further light on the mechanism of oxygen binding by the respiratory pigments.

## SUMMARY.

1. The reactions of oxygen with hæmocyenin take place with the same order of speed as those with hæmoglobin.

2. Under physiological conditions, the oxygen dissociation of *Limulus* serum takes about 1/10 of a second to reach half completion; that of *Maia* takes about 1/25 of a second. The association process is half completed in less than 1/300 of a second for both these pigments. These reactions are too fast to be limiting factors in metabolism.

3. The oxygen dissociation of the blood pigments has been found to be monomolecular, independently of the shape of the equilibrium dissociation curve. This is true, in particular, for sheep hæmoglobin, where the reaction has been followed throughout its entire course with a probable error of about 3 p.c.

4. Increasing the acidity of the blood pigments either greatly increases the rate of oxygen dissociation (*Maia* serum, sheep hæmoglobin, human hæmoglobin), or produces little effect on it (*Limulus* serum). It never much reduces the rate, even on the acid side of the "critical  $pH$ ."

5. Increasing the temperature greatly accelerates the dissociation of *Limulus* hæmocyenin, as it does for sheep hæmoglobin.

6. Under identical conditions, the hæmoglobins of pig, man, sheep, and frog, have quite different rates of oxygen dissociation, as do the hæmocyanins of *Maia* and *Limulus*.

7. The equilibrium constant of dialysed *Limulus* serum as calculated from the rate of dissociation and the rate of combination with oxygen agrees with that obtained by direct measurement.

8. The bearing of these experimental results on the problem of the nature of the oxygen-binding mechanism of the blood pigments has been discussed.

I should like to express my great indebtedness to Dr F. J. W. Roughton, under whose direction this research was attempted, and to the Medical Research Council for defraying part of the expenses. The work was done in the Physiological Laboratory, Cambridge, and during a portion of the time I was aided by a grant from the Coutts Trotter Studentship of Trinity College.

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## OVULATION AFTER BLOOD DILUTION AND CROSS-CIRCULATION.

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### I. INTRODUCTION.

IN an earlier paper [Fee and Parkes, 1929] it was shown that an amount of the ovulation-producing hormone sufficient to cause ovulation is secreted by the anterior pituitary gland of the rabbit within about 1 hour after copulation. Similar results have been obtained by Smith and White [1931] and White [1932]. Brambell and Parkes [1932 b] found that replacement of a considerable percentage of the blood by gum saline at about 1-1½ hours after copulation did not affect the ensuing ovulation, and therefore that an appreciable excess of the hormone over the amount actually required to produce ovulation is present at this time or can be secreted after the dilution. On the basis of experiments carried out at University College, Bangor, this excess or "safety factor" was estimated at rather over 30 p.c. of the amount in the circulation. More recent experiments, carried out in London on rabbits kept under different conditions, suggest a greater margin. Since a certain amount of the ovulation-producing hormone is presumably present in the blood of the non-mated oestrous rabbit, the existence of this large safety factor in the mated rabbit indicated that an adequate amount of hormone should be present in the cross-circulation of a mated and a non-mated rabbit to cause ovulation in both. The experiments on blood dilution, however, suggested that the lowering of the hormone concentration by distribution through two circulations might affect its availability for the ovaries. Actually, we have only succeeded in inducing ovulation in the non-mated partner if the total number of ovaries is reduced by the removal of one from each animal or of both from the mated animal.



## II. TECHNIQUE.

The replacement of blood by gum saline was carried out according to the technique previously described [Brambell and Parkes, 1932 *b*] and was based upon the same calculations. All experiments were carried out under urethane.

The first cross-circulation experiments were performed with the use of chlorazol blue as an anti-coagulant [see Brambell and Parkes, 1932 *a*], but later this technique was abandoned because of the toxicity of the dye, and because the use of an anti-coagulant resulted in the loss of several animals by diffuse hæmorrhage from cut surfaces. The later experiments were carried out by direct anastomosis of the vessels with Crile cannulæ, specially made for the rabbit. Circulation was established from carotid to jugular in all experiments. Direct connection, either by Crile cannulæ or by tubing in association with an anti-coagulant, has the disadvantage that it is difficult to gauge the blood flow, but the use of a reservoir in conjunction with the anti-coagulant was found to present serious difficulties owing to the small blood volume. The cross-circulation was carried out for periods varying from 2 to 4 hours, after which the animals were separated and left for a period adequate for ovulation.

III. THE AMOUNT OF EXCESS OVULATION-PRODUCING  
HORMONE IN THE MATED RABBIT.

The results previously recorded [Brambell and Parkes, 1932 *b*] suggested that the removal of 50 p.c. of the blood 1-1½ hours after copulation, and therefore of 50 p.c. of the hormone in the circulation at that time, inhibited ovulation. It seemed possible therefore that varying the time of removal would give some indication of the time at which the hormone is withdrawn from the circulation by the ovaries. Table I gives the results of 21 experiments carried out on these lines. Both ovaries were left in, except for Exps. 13 and 15. These results show that the surplus hormone circulating or available after copulation is even greater in these experiments than was previously estimated. The technique used was identical with that previously followed in Bangor, and the increased surplus is presumably connected with the strain of rabbit or with environmental conditions.

Attempts to inhibit ovulation by removing 60 p.c. of the blood were abandoned owing to the high mortality following removal of such a large amount, but two of three animals which survived ovulated.

TABLE I. Effect on ovulation of removing 50 p.c. of the blood at various times after copulation.

No. of exp.	Time of removal of blood after copulation		Follicles ovulating
	hr. min.	hr. min.	
12	0 14	to 0 25	10
14	0 20	" 0 31	6
18	0 37	" 0 49	11
17	0 47	" 0 59	14
9	0 40	" 1 10	No ovulation
10	0 54	" 1 10	6
19	0 45	" 1 15	8
6	1 8	" 1 20	No ovulation
11	1 0	" 1 20	4
16	1 0	" 1 30	10
13	1 0	" 1 30	Ovulated
15	1 0	" 1 30	Ovulated
20	1 0	" 1 30	Ovulated
21	1 0	" 1 30	Ovulated
8	1 3	" 1 33	3
4	1 22	" 1 42	4
5	1 22	" 1 52	4
7	1 20	" 2 0	5
2	2 5	" 2 30	5
3	3 0	" 3 30	9
1	3 30	" 4 0	6

The anterior lobe is known to secrete within 1 hour after copulation all the hormone necessary to cause ovulation, and the above results imply that an astonishingly large excess is produced. On the other hand, it is possible that experimentally lowering the percentage of hormone in the blood results in renewed secretion by the hypophysis. Such an action, however, is unlikely in view of the results of the cross-circulation experiments. The apparent excess might also be explained if the ovaries withdrew the hormone from the circulation as fast as it was secreted during the first hour after copulation, though our results on bleeding at this time would still indicate the presence or availability of a large excess. It must be remembered, further, that the ovaries may be withdrawing the hormone from the circulation during the actual time of bleeding.

It is of importance to notice that replacement of 50 p.c. of the blood by gum saline removes not only a proportion of the hormone secreted as a result of copulation, but also some of that already present and concerned in producing the oestrous condition. Since the removal of 50 p.c. of the blood has not lowered the hormone level back to that of oestrus, it must be supposed that the post-copulation concentration is much greater than the oestrous level.

## IV. CROSS-CIRCULATION EXPERIMENTS.

The work described above suggests that it should be easy to secure ovulation in both partners of a cross-circulation between mated and unmated rabbits. Table II gives the results of nine experiments in which both partners lived for at least 15 hours after copulation of the mated partner. Most of the animals survived without difficulty and were killed after about 24 hours.

TABLE II. Cross-circulation of normal oestrous rabbit with mated partner.

No. of exp.	Method of cross-circulation	Ovaries removed before cross-circulation	Time cross-circulation established after copulation of mated partner (min.)	Duration of cross-circulation	Result
1	Chlorazol blue. Reservoir system	One from each animal	36	2 hr. 5 min.	4 r.f. in C.P., none in O.P.
2	"	" "	45	2 hr. 5 min.	3 r.f. in C.P., 3 in O.P.
3	Chlorazol blue. Direct system	" "	45	2 hr. 0 min.	3 r.f. in C.P., none in O.P.
4	"	" "	55	2 hr. 3 min.	No ovulation
5	Crile cannulae	None	c.c. 1 hr. 50 min. after injection of 7.5 c.c. pregnancy urine into one partner	3 hr. 0 min.	Ovulation in both ovaries of both animals
6	"	Both from C.P., one from O.P.	95	3 hr. 0 min.	1 r.f. in O.P.
7	"	Both from C.P.	35	3 hr. 0 min.	7 r.f. in O.P.
8	"	None	29	3 hr. 0 min.	10 r.f. in C.P.
9	"	None	37	3 hr. 20 min.	No ovulation

c.c. cross-circulation.

C.P. copulated partner.

O.P. normal oestrous partner.

r.f. ruptured follicles.

These experiments show that it is possible to induce ovulation in the normal oestrous rabbit by cross-circulation with a mated partner, and thus confirm the general theory of the mechanism of ovulation in the rabbit. On the other hand, greater difficulty has been experienced in obtaining the result than would have been expected from the fact that both the concentration and absolute amount of the ovulation-producing hormone can be much reduced and yet ovulation will occur in the mated animal. It seemed probable that the surplus in the mated animal would have been enough to raise the level in the oestrous animal sufficiently to cause ovulation. In practice, however, we have failed to induce ovulation in the non-mated animal without reducing the total number of ovaries. In addition, our results show that ovulation in the unmated partner is produced most readily when both ovaries are removed from the mated animal as soon as possible after copulation. It would appear that the ovaries begin to withdraw the hormone from the circulation long before

the full amount necessary to cause ovulation has been secreted and that ovaries left in the mated partner withdraw an appreciable amount before cross-circulation can be established.

#### V. SUMMARY.

1. The removal of 50 p.c. of the blood after copulation and replacement by gum saline did not prevent ovulation in a further series of rabbits.

2. This suggests an even greater "safety factor" for the ovulation-producing hormone than was previously found [Brambell and Parkes, 1932 *b*].

3. In spite of this apparent excess, ovulation was produced in the oestrous rabbit after cross-circulation with a mated partner only when the total number of ovaries in the preparation was reduced.

Our very best thanks are due to Prof. F. W. Rogers Brambell and Miss Ruth Deanesly who assisted with many of the earlier cross-circulation experiments.

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## A NOTE ON THE RETINAL ACTION POTENTIAL OF THE HUMAN EYE.

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THE first to record retinal action currents from human subjects were Dewar and M'Kendrick [Dewar, 1877], who observed a deflection of their slow Thomson galvanometer in the same direction as with animals. Kahn and Löwenstein [1924] emphasized the difficulties of the method. They reproduce their best curve, obtained with the string galvanometer, but are not prepared to state that it represents the true form of the retinal response. Hartline [1925] developed the technique and showed that the method might be put to some use in comparing retinal and sensory data. The best records have been obtained by Sachs [1929]. Some of his tracings have been published by Kohlrausch [1931] and are in general agreement with our own. His success is in part attributable to long practise in the avoidance of winking and other movements of the ocular muscles.

Our original intention was to investigate the technique with a view to its possible utilization as a method of comparison with sensory events. An attempt in this direction was, however, carried out only with respect to flicker and fusion. Winks at on and off were found to cause changes of potential very similar to well-known types of retinal action current. A great deal of the time at our disposal was spent in training ourselves as subjects and in establishing the true form of the response under standard conditions. As the records gradually became consistent and repeatable within reasonable limits we decided to take up a simple problem, viz. to compare responses from the central retinal field with responses from peripheral retinal fields under optimal conditions. For such work the human eye is interesting because it is possible in the same retina to compare the action potential from an organ in which cones predominate with that from one in which rods are far more numerous than cones.

## METHOD.

Records from the human subject were obtained with the directly coupled valve amplifier and string galvanometer which have lately been used for registering action potentials in the cat's eye [Granit, 1933; Creed and Granit, 1933]. The subject was seated opposite the stimulating apparatus with his head supported in a padded frame attached to the back of the chair. Leads were taken from the anæsthetized conjunctival sac and from the mouth by means of cotton wicks communicating with Ag-AgCl-Ringer-Locke electrodes. In the course of 20 experiments, each lasting about 30 min., some 330 records were taken.

The standard conditions ultimately adopted were as follows. The subject was in a state of considerable dark-adaptation, the lighting of the room being only such as to enable the experimenter to handle the apparatus. The stimulus was a circular sheet of ground glass illuminated from behind and of about 10 ml. brightness. It subtended about  $7^\circ$  at the subject's eye and carried a cross of luminous paint for central fixation. For peripheral stimulation a piece of faintly illuminated paper  $30^\circ$  to one side was used as a fixation mark. These conditions are optimal in that further increase of area has little effect on the amount of potential developed, increased brightness makes the suppression of winking at the beginning of the stimulus almost impossible, and light-adaptation is accompanied by smaller responses.

## RESULTS.

*The normal response in central and peripheral vision.*

Fig. 1 shows a response which, we are satisfied, represents a very close approximation to the true curve of retinal action potential in the human eye. The silent features of such curves are: absence of *a*-wave, small *b*-wave of about 0.2 millivolt or slightly less, small secondary rise (*c*-wave), and absence of definite off-effect. The only certain difference between central and peripheral responses is the lesser amount of potential developed in the latter. With one observer this difference averaged about 5 p.c.; with the others it was consistently about 15–20 p.c. and thus outside the limits of possible error. The latent period preceding the deflection was never less than  $40\sigma$  and commonly exceeded  $60\sigma$ .

It is interesting to note that the curve of potential is of the same type in the central and peripheral retina. The *b*-wave may have a more definite peak in peripheral vision, but this result was not constant and we

hesitate to accept it as significant. The use of a smaller area, restricting the number of rods participating in the central response, was found to be of little service in our attempt to compare central and peripheral effects. The responses then became too small.

*Comparison with the cat's retinal action potential.*

It is interesting to compare Fig. 1 with the response of the cat's eye when leads are taken from the eyeball and from the decerebration wound [see Granit, 1933] under otherwise similar conditions. The latter shows

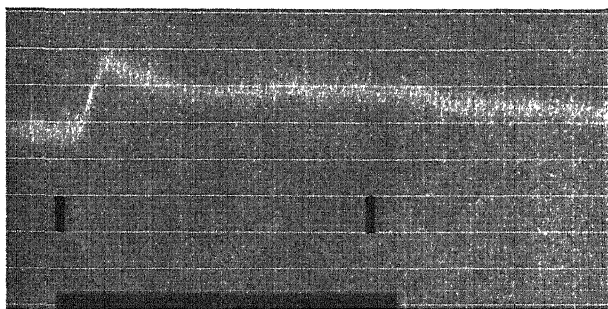


Fig. 1. Curve of retinal action potential for observer R. G. with central vision. Diameter of stimulating disc about  $5^{\circ} 30'$  of visual angle. The  $b$ -wave deflection is 0.15 millivolt. For general conditions see text. Time marker gives  $100\sigma$  and  $20\sigma$ . An interval of 1 sec. is marked in ink. Stimulus directly photographed. Observer unshielded and picking up quick oscillations which broaden the contour of the string.

a small  $a$ -wave, a  $b$ -wave with a sharp peak rising to about 0.5 millivolt, a much larger  $c$ -wave, and a more definite off-effect. It is difficult to believe that the human eye is so much more efficiently shunted as to account completely for these differences. A more satisfactory explanation is provided by the fact that the cat's curve of retinal action potential evoked by a stimulus one hundred times less bright resembles, both in shape and size, the human response of Fig. 1. It is therefore probable that the human eye is pitched to a higher level of stimulation and that more light is required to produce a given response. The nocturnal habits of the cat lend support to this suggestion. The human response is not significantly altered by using nose, temporal bone, or forehead instead of mouth for the indifferent electrode.

An alternative possibility, which would explain both the smallness of the  $b$ -wave and the long latent period in the human eye, is that the

negative component P III [Granit, 1933] of the action potential may be larger than in the cat and may hinder the deflection caused by P II. The absence of  $\alpha$ -wave and of marked off-effect make this suggestion unattractive.

*Flicker and retinal potentials.*

Observations with intermittent light were made by Sachs [1929] on his own eye. The records from his work published by Kohlrausch [1931] are, however, all responses to continuous stimulation. The disappearance of the sensation of flicker is stated to be accompanied by the disappearance of ripples in the electrical record. We have tried to determine how closely retinal and sensory data could be correlated in this way. It was com-

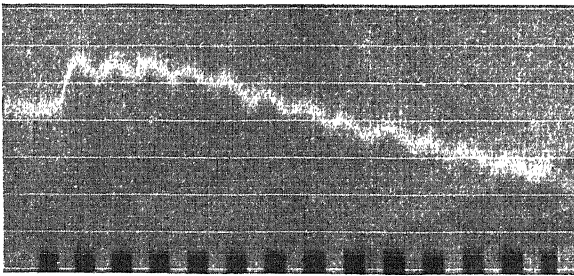


Fig. 2. Retinal action potential of observer R. S. C. Intermittent light at about eight flashes a second. Standard conditions. Considerable base-line drift.

paratively easy (Fig. 2) to confirm the fact that very coarsely flickering stimuli give wavy responses. But it was found impossible, owing to the smallness of the oscillations and the unsteadiness of the base line, to determine accurately the "fusion point" in the records. An obviously flickering stimulus would give a tracing indistinguishable from one obtained with steady illumination. This does not imply that retinal and sensory "fusion" occur at different rates of alternation. Indeed it seems certain on other grounds that the retinal frequency cannot be lower than the sensory, and there is some reason for believing them to be closely related [Creed and Granit, 1933]. Our experiences described above only indicate that the question whether this relation is one of identity cannot be solved by this technique. Even with elaborate shielding of the observer and increased amplification, it is unlikely that a sufficiently steady base line could be obtained.



## DISCUSSION.

The fact that the peripheral response is similar in type to the central one, but develops less potential, is worth commenting upon. In the periphery of the retina rods are nearly twenty times as numerous as cones [Chievitz, 1889; Fincham, 1925]. It is therefore safe to assume that a much greater number of rods have been stimulated there than in the centre, even though the latter area was certainly not rod-free.

The amount of potential developed may depend upon four factors: (i) the sensitivity of the receptor (thus it increases with dark-adaptation), (ii) the number of receptors participating in the reaction (studied by Graham [1932] in an eye lacking internuncial neurones), (iii) synaptic interaction [Granit, 1933], (iv) size of negative wave. If the potential were developed in the rods and cones themselves, only the first two of these factors would come into play. The high sensitivity of rods as compared with cones in the dark-adapted eye would then presumably result in larger action potentials from the periphery than from the centre of the retina. The reverse, however, is found to be the fact. The available evidence, summarized by Granit [1933], indicates that the potential is developed in the retinal synapses or neurones, proximal to the rods and cones. Thus we are led to consider factor (iii), which at present is difficult to evaluate. According to Chievitz [1889] there are about 80 receptors for each ganglion cell  $21^\circ$  from the fovea, whereas in the fovea itself the ratio is 1 : 1. Far fewer bipolar and ganglion cells are therefore involved in our peripheral than in our central responses, and this may account for the difference observed. We cannot say to what extent sensitization of the rods and synaptic interaction may compensate in the periphery for a decreased number of nerve cells, but the explanation of our findings which has just been outlined is in keeping with other evidence as to the site at which retinal action potentials are developed. In view of the similarity of form between central and peripheral responses, there is unlikely to be any significant difference in the relative sizes of P II and P III.

## SUMMARY.

1. The retinal action potential of the human eye has been recorded with a string galvanometer and a directly coupled amplifier under standard conditions.

2. Responses from the periphery of the retina ( $30^\circ$  from the fixation point) resemble those from the centre in general features, but less potential is developed. The significance of this is discussed.

3. Responses to intermittent illumination at slow rates of alternation show corresponding ripples in the electrical record. It is not possible to correlate the findings directly with sensory data on fusion frequency.

We take this opportunity of thanking the Rockefeller Foundation for a grant (to R. G.) for apparatus, and the Christopher Welch Trustees for a grant (to R. S. C.) towards the expenses of photographic material.

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## ALBUMINURIA IN THE NORMAL MALE RAT.

By MURIEL E. BELL<sup>1</sup>.

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University College, London.)*

DURING the course of some feeding experiments an observation was made regarding the physiology of the rat to which no more than a passing reference in the literature can be found, and which, therefore, seemed worth recording. The experiments, the results of which are to be published shortly, were designed primarily to test the extent to which the growth-retarding effects of diets rich in protein could be overcome by the addition of alkali. But also, as it had been reported by Newburgh and Curtis [1928], Newburgh and Johnston [1931], Blatherwick, Medlar, Connolly and Bradshaw [1931], that kidney damage occurred if rats were given diets containing a large proportion of protein, it was decided to see whether the diets were nephropathogenic. Since, in addition to the histological evidence, these authors found increasing albumin and casts, the test animals were put into Hopkins cages in order that the urine might be tested for albumin. In the experiments in which they observed greatest damage the protein given was meat, whereas, for the sake of keeping our growth curves comparable with those of former experiments from this laboratory, casein was used.

## EXPERIMENTAL.

The various groups of animals being studied were ingesting diets containing normal and supernormal amounts of casein, with and without additional alkali. Each group was put into a metabolism cage; the food was mixed with water into a thick paste, so that the urine might be collected with as little contamination from the food as possible. For the first few weeks of the experiment it was observed that there was no albumin in the urine collected from any of the groups. Later, however, each group exhibited albuminuria. This was the case whether the diet

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contained 20 or 70 p.c. protein, whether alkali to reduce the acidity of the urine had been added or not; it occurred alike with stock rats on stock diet as with experimental rats. In order to determine what percentage of the animals manifested this albuminuria, they were each put separately into the metabolism cages, and were each observed over a period of several days. It was noticed that about half the individuals in each group excreted albumin. When a fair number had been tested, it became apparent that whereas the urine of females was usually free from albumin, the urine of males which had reached maturity invariably contained a substance which formed a coagulum on boiling the urine and acidifying with a weak solution of acetic acid. The substance did not precipitate with acetic acid alone; the application of heat was necessary. Of a total of 70 rats examined, the 34 bucks were all excreting albumin, 33 females gave negative tests, three does showed a small amount, and of these three does, two were ingesting normal diets. There was still a positive test in the urine of males even when they had been separated from the does for several days. There being an obvious sexual factor, some of the specimens from males were examined for spermatozoa, which were present in several but not all of those investigated. We concluded, therefore, that as the source of the protein was apparently not the testes or seminal vesicles, it originated from some accessory gland such as the prostate.

The quantity present was in the vicinity of 0.5–0.8 g. per litre, as measured in an Esbach albuminimeter. No attempt was made to determine the class to which this coagulable protein belonged; it is here called "albumin" simple in the clinical sense. This constituent began to appear usually when the rats were 100–120 g. in weight, so that its incidence seemed to be connected with the occurrence of puberty. The presence of albumin in the urine is less surprising when one realizes that the rat's seminal fluid after ejection gives rise to a spontaneous coagulum known as the "vaginal plug."

An attempt to collect samples from the bladder at autopsy did not meet with enough success to justify deductions, though the few samples obtained did not give the test for albumin. In one rat, however, an anomalous structure was noticed—a spiral, gelatinous-looking structure near the neck of the bladder; and when this was teased out under the microscope, it was found to be a collection of spermatozoa, and the urine from the bladder contained sperms. We are at a loss to know what anatomical relationships in the genito-urinary system of the rat allow of the backward migration of spermatozoa into the bladder. It is possible that the relaxation of the sphincter under chloroform allows secretion to

pass back, but it does not explain the presence of the well-formed structure observed on this one occasion. In another rat, a mass, macroscopically similar, was found, but under the microscope it was amorphous. It will be realized how readily a stone can be formed in the bladder with such a foreign body as a nucleus.

From the foregoing experimental evidence, we believe that inferences as to damage of the kidney cannot reasonably be made from the detection of albumin in the urine of the mature male rat, unless it exceeds the normal amount. The quantity found by Newburgh in the urine of his experimental animals varied from 0 to 3.0 p.c. Newburgh and Curtis [1928] state that "the acidified urine from normal animals often becomes opalescent or even cloudy on being boiled," but make no distinction between urines of males and females. Longwell, Hill and Lewis [1932] observed that, in animals which were receiving excessive amounts of cystine, "there was at all times a slight albuminuria which... was no more than is normal for the male rat." In tables quoted by Donaldson [1924], the figures for nitrogen partition in the urine do not imply any difference between the urines of the two sexes, and no account is taken of protein excretion.

#### SUMMARY.

A protein coagulable by heat is present in the urine of normal male rats after they have reached maturity. The amount excreted is about 0.5-0.8 g. per litre.

Grateful appreciation is herewith expressed to the Royal Society of Medicine for the scholarship which has enabled me to make these observations; to the Medical Research Council for defraying cost of materials; and to Prof. J. C. Drummond for helpful advice and criticism.

It has been brought to the notice of the author since this paper was sent for publication that similar observations have recently been published by Addis, but up to the time of going to press, no copy of the Journal containing his results has been available.

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## THE DIFFUSION OF LACTATE INTO AND FROM MUSCLE.

By S. C. DEVADATTA.

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CERTAIN constituents of the voluntary muscles of the frog are able to diffuse out of the muscle into a surrounding saline, and can also diffuse into the muscle if previously dissolved in the saline in a concentration sufficiently high<sup>1</sup>. There exists in such cases a certain concentration of the substance in saline which will be in equilibrium with the tissue. This critical concentration provides a measure of the concentration of the substance in the tissue—or rather in such part of the tissue as is concerned in the diffusion. In the case of creatine this measure of the concentration in the tissue was found by Eggleton [1930] to agree with values obtained by Dulière [1929] by direct analysis. This indicates that the partial osmotic pressure is the factor controlling the direction and extent of diffusion. Similar indirect determination of the concentration of phosphate in muscles [Stella, 1928] gave results rather lower than those obtained by direct analysis; but it must be remembered that direct estimations of the phosphate in resting muscles are liable to yield high results on account of the breakdown of phosphagen at the moment of death.

The present communication presents experiments leading to the conclusion that the concentration of lactate in both resting and fatigued muscles can be measured by the indirect "counter-diffusion" method, since the values obtained agree well with those in the literature obtained by direct analysis.

### TECHNIQUE.

The procedure adopted was that used by Eggleton for determining the concentration of creatine in muscle. One of a pair of resting gastrocnemii was placed in a Ringer's solution containing, for example, no lactate,

<sup>1</sup> Concentrations recorded in this paper were measured in mg. per 100 g. of solution; for calculation purposes these should be expressed in mg. per 100 g. water. For Ringer's solutions, however, the correction is small, and was neglected.

and the other in a solution containing, say, 60 mg. per 100 g. The amount of solution used was the same in both cases, about one and a half to two times the weight of the muscle. Gentle stirring was maintained by bubbling a suitable gas through the solution. Air and nitrogen respectively were used for experiments on resting and fatigued muscles, at a constant rate in all the cases. The tubes containing the muscle and solution were surrounded by ice in a beaker to prevent the formation of lactic acid; all the experiments were carried out at 0° C. After 2½ to 3 hours a known amount of the solution was analysed for lactate. The concentration of lactate in the solutions before diffusion was also determined. As shown by Eggleton, the equilibrium concentration of lactate in the muscle can be calculated from the formula:

$$C = \frac{L_0 l_1 - L_1 l_0}{(L_0 + l_1) - (L_1 + l_0)}, \quad \dots\dots(1)$$

$C$  = Equilibrium concentration in mg. per 100 g.

$L_0$  = Initial concentration in "high lactate" Ringer's solution.

$L_1$  = Final concentration in "high lactate" Ringer's solution.

$l_0$  = Initial concentration in "low lactate" Ringer's solution.

$l_1$  = Final concentration in "low lactate" Ringer's solution.

According to A. V. Hill [1930] the concentration of sodium chloride in a Ringer's solution isotonic with resting muscle is 0.71 p.c. This concentration was employed. For fatigued muscle 1.2 p.c. of sodium chloride in Ringer's solution was found to be isotonic with muscle. The concentration of potassium chloride and calcium chloride were 0.014 and 0.0125 p.c. respectively. In order to keep the pH of the Ringer's solution at 7.1, a buffer solution of 0.2 molar phosphate containing 65 p.c. of disodium hydrogen phosphate and 35 p.c. of sodium dihydrogen phosphate was prepared; 20 c.c. of this solution were added to 980 c.c. of Ringer's solution, giving a concentration of 12 mg. phosphorus per 100 c.c. For higher or lower concentrations of lactate, an equivalent amount of sodium chloride in the Ringer's solution was replaced by sodium lactate, so that the osmotic pressure of the Ringer's solution in high or low lactate solutions was the same.

Sodium lactate at pH 7.1 was prepared by myself. Lactic acid was diluted and boiled for 8 hours to convert all lactic anhydride into lactic acid. The solution was neutralized with caustic soda to pH 7.1, the indicator (phenol red) being used externally, lest it should have any toxic effect on the muscle cells. The diffusion was carried out at 0° C. for three hours in all cases.

Lactate in the solution was estimated by Clausen's [1922] method modified by Friedemann, Cotonio and Shaffer [1927], but without aeration [Meyerhof, 1930].

#### RESTING MUSCLE.

In order to obtain maximum quantities of lactate for estimation, muscles of the thigh were chosen. The frog, previously cooled to 0° C., was rendered insensible by a blow on the head or by decerebration, and sectioned immediately above the iliac bones. The abdominal muscles and the viscera in the pelvis were removed, and the skin was carefully dissected off. The legs were separated without injury to the muscles by section of the os pubis with a razor.

TABLE I. Concentration of lactate in resting frog muscle.

Exp.	Weight of muscles in g.		Lactate in mg. per 100 g. of Ringer's solution		Equilibrium calculated (mg. per 100 g.)
	Initial	Final	Initial	Final	
1. <i>l</i>	10.35	10.35	0	7.2	30
<i>L</i>	10.36	10.35	71.6	61.0	
2. <i>l</i>	12.48	12.49	0	6.1	32
<i>L</i>	12.49	12.50	67.7	61.0	
3. <i>l</i>	13.95	13.96	0	5.0	23
<i>L</i>	13.87	13.87	67.7	58.4	
4. <i>l</i>	16.40	16.40	0	3.0	27
<i>L</i>	16.32	16.35	71.0	65.0	
5. <i>l</i>	16.50	16.51	0	7.7	26
<i>L</i>	15.36	15.35	79.2	63.4	
6. <i>l</i>	15.97	16.02	0	7.2	29
<i>L</i>	15.94	15.97	37.7	36.0	
7. <i>l</i>	4.71	4.65	0	6.4	24
<i>L</i>	4.50	4.57	37.7	31.1	
8. <i>l</i>	4.35	4.35	0	9.9	17
<i>L</i>	4.35	4.35	37.7	26.4	
9. <i>l</i>	1.36	1.37	0	6.2	24
<i>L</i>	1.39	1.34	44.9	39.7	
10. <i>l</i>	1.83	1.83	0	3.0	13
<i>L</i>	1.84	1.85	37.0	31.5	
11. <i>l</i>	1.22	1.23	0	4.0	21
<i>L</i>	1.23	1.24	37.0	34.0	
12. <i>l</i>	1.45	1.45	0	5.3	18
<i>L</i>	1.25	1.24	37.0	31.6	
13. <i>l</i>	0.98	0.98	0	4.5	16
<i>L</i>	0.99	0.98	39.4	33.3	
14. <i>l</i>	2.60	2.60	0	3.0	23
<i>L</i>	2.65	2.64	46.0	43.0	
15. <i>l</i>	2.84	2.83	0	2.5	14
<i>L</i>	2.90	2.89	47.2	41.1	

*l*, low lactate Ringer's solution; *L*, high lactate Ringer's solution. Exps. 1-6 were performed on thigh preparations. Exps. 7-15 were performed on gastrocnemii, or calf preparations.



Consistent results of 30 mg. per 100 g. of solution were obtained for the concentration of lactate in equilibrium with frog muscle. To confirm this, muscles were placed in a modified Ringer-lactate solution containing 30 mg. lactate per 100 g. of solution, and it was found that the muscles were in equilibrium with regard to lactate; the concentration of lactate in solution remained constant. This figure, 30 mg. per 100 g., is twice the amount of the accepted figure for resting frog muscle. This was thought to be probably due to partial asphyxia of the muscles, or due to the dissection of the recti muscles which are attached to the skin; even though the injured fibres were cleared away there might be fibrillar contractions taking place, giving rise to lactic acid in the muscle. Therefore they were abandoned and gastrocnemii, preferably with their bony attachments, were selected; in other cases the second segment of the leg with the muscles of the calf were taken. Both these preparations can be made without any injury to the muscles concerned. The diffusion technique was unaltered. The preparations were reweighed at the end of each experiment and found to have neither gained nor lost in weight; therefore the solution was isotonic. For equation (1) to be valid, there should be no transference of water into or from the muscle. The muscles were stimulated and found to be excitable. From each tube 2 g. of the solution were taken and analysed for lactate.

An average of the results recorded for Exps. 7-15 shows a concentration of 20 mg. per 100 g. of solution. From this figure, it is easy to arrive at the concentration of lactate in the muscle. Since the muscle contains 80 p.c. by weight of water the concentration of lactate in the muscle is 16 mg. per 100 g. muscle.

#### FATIGUED MUSCLE.

In this case the gastrocnemius muscles of the frog were fatigued by causing them to contract isometrically. They were dissected out, weighed and introduced into the tubes containing modified Ringer-lactate solutions. In order to prevent oxidative recovery the solution was covered in the earlier experiments with a layer of liquid paraffin. The steady gentle stirring was maintained by bubbling nitrogen through the solution. The use of liquid paraffin renders it impossible to find if the weight of the muscle had remained constant during the experiment; but the muscles could be tested to see if they were still excitable. Later on the use of liquid paraffin was given up and the tubes were stoppered, after the air had been displaced by nitrogen. The weight of the muscles in these cases

was noted and found to be constant. At the conclusion of the experiments the muscles were found to be still excitable.

TABLE II. Concentration of lactate in fatigued frog muscle.

Exp.	Weight of muscles in g.		Lactate in mg. per 100 g. of Ringer's solution		Equilibrium calculated (mg. per 100 g.)
	Initial	Final	Initial	Final	
1. <i>l</i>	—	—	0	22	232
<i>L</i>	—	—	512	487	
2. <i>l</i>	—	—	0	25	220
<i>L</i>	—	—	500	470	
3. <i>l</i>	—	—	0	23	230
<i>L</i>	—	—	502	476	
4. <i>l</i>	1.24	1.25	0	16	270
<i>L</i>	1.25	1.25	502	489	
5. <i>l</i>	1.62	1.62	0	14	214
<i>L</i>	1.64	1.63	412	399	
6. <i>l</i>	1.36	1.39	0	24	218
<i>L</i>	1.37	1.36	412	391	
7. <i>l</i>	0.92	0.96	11	34	214
<i>L</i>	0.90	0.90	418	394	
8. <i>l</i>	1.43	1.44	11	30	205
<i>L</i>	1.50	1.49	418	396	
9. <i>l</i>	1.09	1.10	11	36	286
<i>L</i>	1.09	1.09	418	406	

The average equilibrium concentration is 238 mg. per 100 g. of solution. This corresponds to a concentration of 190 mg. of lactate per 100 g. of fatigued frog muscle.

#### SUMMARY.

Studies of diffusion of lactate into and out of resting and fatigued frog muscles indicate an apparent concentration of lactate in the water of the muscle of 20 and 238 mg. per 100 g. respectively. This is in good agreement with the values obtained from direct analyses of such muscles by other workers. It follows that all the water of the muscle is available to dissolve lactate.

The expenses of this work were defrayed by a grant from the Moray fund. I am grateful to Dr P. Eggleton for helpful suggestions.

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AN ISOLATED MAMMALIAN HEART PREPARATION  
CAPABLE OF PERFORMING WORK FOR  
PROLONGED PERIODS.

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IN experiments performed to determine how far the lungs influence the increased diastolic filling of the heart as a result of a reduction in respiratory (intrathoracic) pressure, it was found necessary to replace the lungs of a heart-lung preparation by a blood oxygenation apparatus [Daly, 1927]; for the oxygenator a modified form of the apparatus designed by Hooker [1915] and Drinker [1922] was used. In such an isolated heart preparation performing work, it was found that the heart quickly became hypodynamic in spite of good oxygenation of the blood, the average duration of five experiments being only 36 min. (limits 25–60 min.). This rapid onset of cardiac failure, as compared with the heart-lung preparation which lasts at least five hours if not overworked, was also observed independently by Verney [1927], and has since been observed by others. At that time no attempt was made to investigate the cause of the heart failure in the isolated heart preparation, but recently we have carried out a number of experiments which have enabled us to maintain the isolated heart in good condition for a period of time at least equal to that generally obtained with the heart-lung preparation and also have disclosed conditions which may be of interest relative to all perfusion experiments performed with defibrinated blood.

We do not propose to describe in detail the various forms of apparatus used in some twenty-four experiments before final success was obtained, but will merely mention briefly the cause of our early failures and particularize those experiments which are relevant to the points we wish to discuss.

METHODS.

In all our experiments a Starling's heart-lung preparation was made to run on defibrinated blood and then converted to an isolated heart preparation performing work by replacing the lungs with some type of

Hooker-Drinker blood oxygenating apparatus. In order to do this a cannula was inserted into the central end of the pulmonary artery or into its left branch and the blood conducted to the oxygenator and finally back

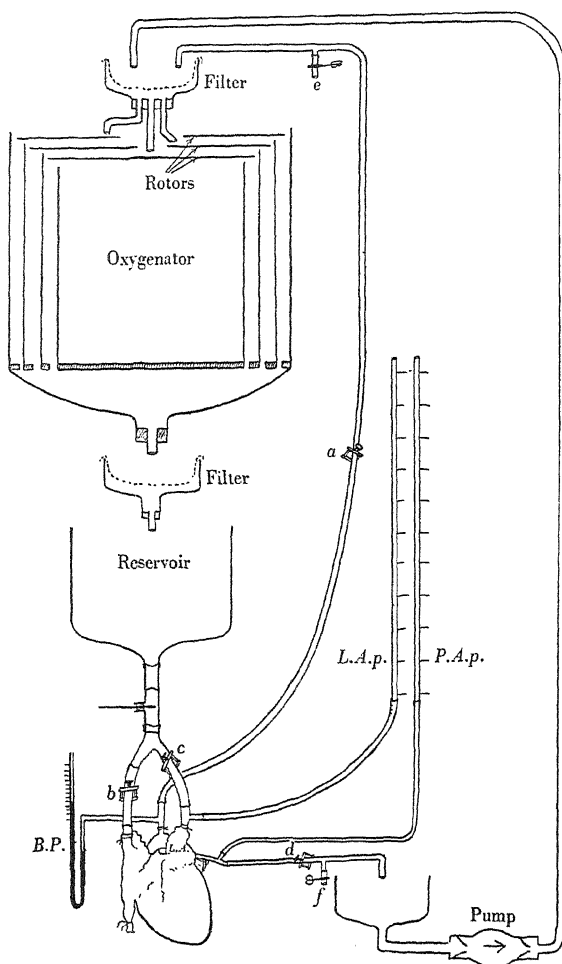


Fig. 1. General arrangement of apparatus.

to the left auricle through a cannula tied into the left auricular appendix. The lung roots were then tied off and the lungs removed. Owing to the height of the oxygenating apparatus it was necessary to insert a pump to lift the blood from the level of the heart to the top of the apparatus, thus saving the right heart from having to put out blood at a pressure above

the normal. The position of the oxygenator relative to the heart was not always the same in every experiment, but, whatever the arrangement, the oxygenation of the blood from the right heart, at a normal or less than normal pulmonary arterial pressure, was always achieved. It is convenient at this stage to describe in detail the method finally adopted and later to indicate the necessity for taking certain precautions to ensure success.

In all experiments dogs were used, anaesthetized with 0.1 g. per kg. body weight of chloralose injected intravenously after induction with chloroform and ether, except in the last two experiments in which the heart-lung preparation animal and the dogs from which blood was collected were anaesthetized with 0.04 g. per kg. body weight of "Nembutal" injected intravenously under local anaesthesia without induction with chloroform and ether. The blood collected for the experiment is defibrinated and filtered through a tower of four or five cambric filters and then kept at 37–39° C. until ready to be used; it is filtered again just before use. The cambric filters had a mesh, measured dry, of approximately  $50 \times 100 \mu$ . A Starling's heart-lung preparation is made and arranged so that the left ventricle pumps the peripheral systemic blood to a cambric filter placed above the oxygenator (Fig. 1). The blood then drains through the oxygenator on to a second filter thence to a reservoir which has a capacity of 1.5 litres and is placed in a thermostat. A screw clip (*a*) on the rubber tubing is used for adjusting the systemic pressure in the region of 110 mm. Hg. A cannula with a side tube for taking the left auricular pressure is inserted into the left auricular appendix and connected by a Y-piece to the reservoir outflow tube, a screw clip (*c*) being placed tightly on the rubber connection. By this arrangement the blood from the reservoir may be diverted either to the right or to the left auricle. Pure oxygen is then passed through the oxygenator and the rotors set in motion, the screw clip (*c*) being open and screw clip (*b*) closed so that the blood from the reservoir flows into the left auricle and ventricle. Thus, the peripheral systemic blood is oxygenated by the oxygenator and only the coronary blood by the lungs before it mixes with the reservoir blood in the left auricle. Positive pressure artificial respiration is carried out with the Starling Ideal respiration pump. This concludes the first stage of the operation.

In the second stage, a cannula with a side tube is inserted centrally into the left pulmonary artery, both lung roots are ligated and the lungs removed; the coronary blood which passes out from the left pulmonary arterial cannula is collected in the reservoir of a Dale and Schuster [1928] pump regulated to the coronary flow, and conveyed to

the filter above the oxygenator. The side tube of the pulmonary cannula is connected to a manometer so that the pulmonary arterial pressure may be taken, and a screw clip (*d*) enables this pressure to be maintained at any

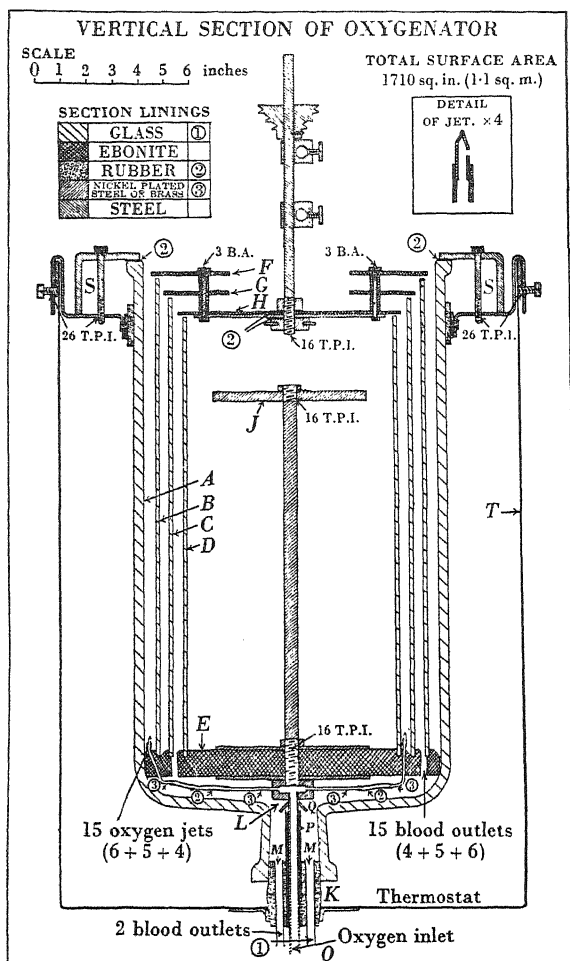


Fig. 2. Oxygenator.

desired level. By adjustment of the screw clips *b* and *c* the total quantity and the proportion of blood dealt with by each side of the heart can be adjusted at will. Since the blood-pressure of each side of the heart can also be adjusted separately, the quantity and type of work performed by each

side of the heart can be varied as desired. The blood flows are measured through the side tubes *e* and *f*, but in a few experiments we recorded blood flows with Pitot tubes. When both screw clips *b* and *c* are open, the blood flow from the left pulmonary artery consists of the amount flowing from the reservoir into the right auricle plus the coronary flow. In order to measure the coronary flow under these conditions it will be necessary to measure the flow from the side tube *f* and subtract from it the amount coming from the reservoir into the right auricle obtained by insertion of a flow recorder at *b*. For the purpose of comparing one experiment with another the peripheral systemic flow and resistance are kept approximately constant and the left auricular pressure is taken as the criterion of the functional capacity of the heart. Except for a small slit to allow of insertion of the left auricular cannula, the pericardium is kept intact.

Our early experiments had indicated the need for not only a larger oxygenator but one which would be able to deal with sudden changes in flow. Since existing forms of oxygenators at that time were either too small or unable to deal satisfactorily with a fluctuating flow it was decided to design an oxygenator sufficiently large to deal with flows much greater than we anticipated for the present experiments. Fig. 2 is a scale drawing of this apparatus which is in effect three Hooker-Drinker oxygenators arranged concentrically. The blood is distributed by three jets on to the ebonite discs *F*, *G*, *H* and spun out on to the walls of the glass cylinders *A*, *B*, *C* respectively. The films of blood flowing down these cylinders are oxygenated by oxygen from 15 jets (6 for *A*, 5 for *B* and 4 for *C*) mounted in the ebonite base *E*. A similar number of holes in this base serve as blood outlets to the bottom of the bell jar *A* and hence to the blood reservoir by the two glass tubes *M*, *M'*. The cylinder *D* is to prevent waste of oxygen in the centre of the apparatus. The oxygen passes into the apparatus through the tube *O* to the jets by way of the distributor *L* (Fig. 3). The tube *O* slips into the tube *P* mounted in the rubber bungs *K*. The base *E* and the three cylinders *B*, *C*, *D* can thus be removed from the bell jar *A* by the handle *J* (Fig. 4). *Q* is a rubber baffle to prevent any blood spray escaping between *O* and *P*.

The apparatus is heated by immersing the jar *A* in water at 38° C. in the galvanized tank *T*, the jar being held down by the clamps *S*. The water is heated by three 500-watt electric immersion heaters controlled by resistances, and stirred with two propellers not shown. The dimensions of the apparatus can be seen from the scale drawing and it should suffice to mention that the bell jar *A* is 18½ in. high and 11¼ in. inside diameter.

The inner surfaces of *A*, *B*, and *C* are respectively 650, 570 and 490 sq. in., *i.e.* a total surface area of 1710 sq. in. (1.1 sq. m.). The apparatus is capable of dealing with relatively large volumes of blood. While not intended to cope with flows much over 600 c.c. per min. a sudden gush of blood of even as much as 2500 c.c. per min. could be accommodated without overflowing. The volume of blood pooled in the apparatus at a

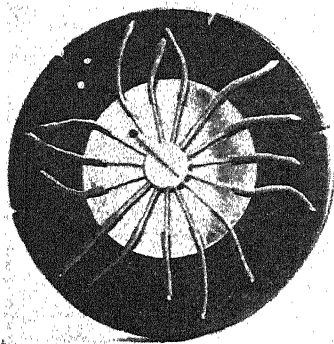


Fig. 3.

Fig. 3. Distributor of oxygenator.

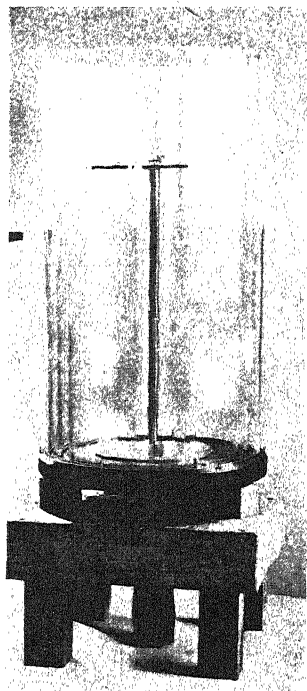


Fig. 4.

Fig. 4. Inner cylinders of oxygenator.

flow of 660 c.c. per min. is 205 c.c. using cylinders *A* and *B* only, 300 c.c. using all three cylinders and 350 c.c. at 850 c.c. per min. All metal parts of the apparatus are heavily nickel plated. The apparatus was *completely* dismantled and cleaned after each experiment with soap and water and rinsed with plenty of clean water, and before each experiment the filming surfaces were treated with cotton wool and spirit.



## RESULTS.

*Preliminary experiments.*

The technique of our earlier experiments was essentially the same as described except that the defibrinated blood was first filtered through muslin and then through one cambric filter of  $100 \times 200\mu$  mesh when measured dry, and no filters were inserted above the oxygenator reservoir. Our results confirmed those of Daly and Verney in that cardiac failure associated with a rise in left auricular pressure occurred early on in the

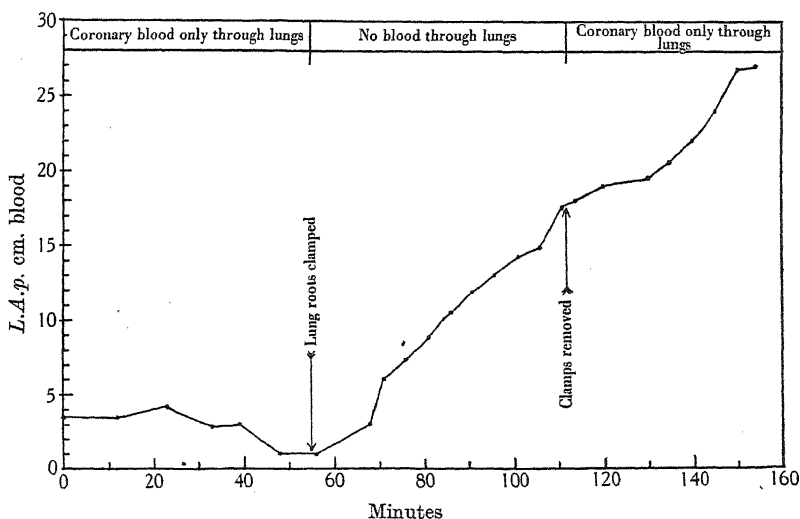


Fig. 5. Effect on left auricular pressure of changing from a heart-lung preparation, in which only the coronary blood flows through the lungs, to an isolated heart preparation. Coarse cambric filters used.

experiment. In searching for a cause of this failure we tried stopping the operation at the end of the first stage so that only the coronary blood flowed through the lungs, the peripheral systemic blood passing through the oxygenator. It was then found that the left auricular pressure remained nearly constant for at least one hour and a quarter, this being the longest period tried, but on performing the second stage of the operation and switching over to the isolated heart preparation, the venous pressure started to rise rapidly. In this series of experiments instead of ligating and removing the lungs for the second stage of the operation we used gastro-enterostomy clamps guarded with rubber tubing for cutting off the pulmonary circulation. This procedure had the

advantage of enabling us to return to the conditions of the first stage by removing the clamps and clipping the rubber tubing on the pulmonary arterial cannula. Fig. 5 shows the effect of running the preparation with only the coronary blood going through the lungs and then changing over to the isolated heart preparation and finally back again to the initial conditions. It is clear that changing over to the isolated heart preparation rapidly diminished the functional capacity of the heart as evidenced by the rise in venous pressure, moreover a return to the initial conditions did not restore the efficiency of the heart. In these experiments the blood was collected from the right heart by a cannula inserted into the pulmonary artery, and it was thought that this might be responsible for the early onset of cardiac failure, but a control experiment in which the pulmonary artery was cannulated in the same way, but the blood allowed to return to the left auricle through the lungs, lasted 5 hours. We were left therefore to seek some other cause of the failure. Owing to the good colour of the blood throughout these experiments we felt reasonably certain that anoxæmia was not the cause of the rapid rise in venous pressure, although at this stage of the investigation no blood oxygen determinations were made.

In the light of these experiments it was natural to speculate as to how far the lungs might be responsible for the addition to or removal from the blood of one or more substances necessary for the proper functioning of the heart. Such a mechanism might be a purely mechanical one, the pulmonary vascular bed acting merely as a filter of abnormal blood constituents formed during blood defibrination, during blood incubation or in its passage through the oxygenator, or again it might be a chemical one, and we were at once attracted to the hypothesis that histamine addition to or removal from the blood by the lungs might play some part. We had in mind the investigations of Best, Dale, Dudley and Thorpe [1927] which demonstrated the presence of large quantities of histamine in the lungs, the significance of which is as yet unknown. Our results, however, gave no support to the view that lack of histamine is responsible for the hypodynamic condition of the heart. In one experiment 0.05 mg. histamine per minute was added to the blood of the isolated hypodynamic heart preparation for 14 min., and in a second experiment 0.06 mg. per minute for 23 min., without any apparent effect on the heart. The method of adding the histamine to the blood by continuous infusion renders it difficult to compare our results with those of other workers. In experiments on the heart-lung preparation, Fühner and Starling [1913] obtained marked dilatation of the heart succeeding a transient volume

diminution, with a dose of 0.5 mg. histamine injected into the pulmonary artery; Rühl [1929] and Rössler [1930] obtained similar results with larger doses (0.5–2.0 mg.); Müller, Salomon and Zuelzer [1932] found no evidence of cardiac damage by the addition of single doses of less than 0.1 mg. histamine. Larger doses, 0.5 mg., damaged the fresh heart but not the heart which had previously been treated with successive small doses of histamine.

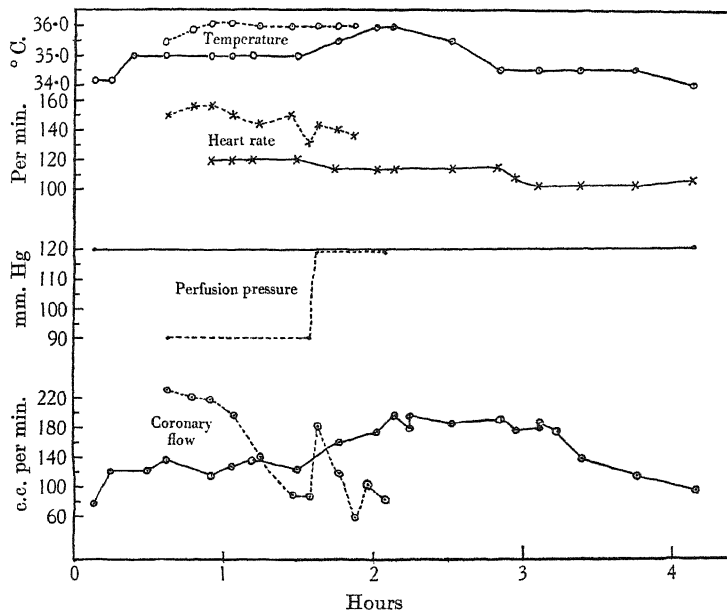


Fig. 6. Isolated perfused heart. (a) The broken lines indicate the events taking place when the blood is filtered through coarse cambric. (b) The continuous lines, the events when the blood is filtered through fine cambric. In (a) the coronary blood flow shows a rapid and progressive diminution.

The histamine content of the hearts after perfusion or after performing work as a heart-lung preparation or heart-oxygenator preparation showed no significant departure from the values obtained from normal hearts.

#### *Isolated perfused heart.*

Attention was then directed to the question of blood filtration. Up to this time the blood had been filtered immediately after defibrination through one layer of muslin and a layer of cambric having a mesh of  $200 \times 100\mu$  when measured dry. In general it was filtered again through

cambric just before use. Using this method of blood filtration we set up an isolated heart preparation perfused through the aorta with a Dale and Schuster pump. Cannulae were placed in the left ventricle through the left auricle, in the central end of the pulmonary artery and in the right auricle to enable the coronary blood and any blood finding its way past the aortic valves to have a free exit. An oxygenator was used for oxygenating the blood. The coronary blood was collected from the right auricle and pulmonary artery. The result of one experiment is shown in Fig. 6*a*, and it will be seen that the coronary blood flow at constant perfusion pressure gradually diminished, and a rise in perfusion pressure towards the end of the experiment increased the coronary flow only temporarily. This is in contradistinction to the heart-lung preparation in which the coronary blood flow, if anything, generally increases as the experiment progresses if the mean systemic pressure is kept constant. A second experiment was equally unsuccessful in warding off the cardiac failure, so precautions were taken to filter carefully the defibrinated blood before and during the perfusion of the heart. The blood was first filtered through coarse muslin and then through five cambric filters of approximately  $50 \times 100 \mu$  mesh; a similar fine filter was inserted in the perfusion circulation. With these precautions it was found that the heart beat strongly for over 4 hours, the blood flow showing no evidence of diminution (Fig. 6*b*). Our results emphasize the importance of the warnings issued by Brodie [1903] and especially by Anrep and Häusler [1928] in relation to coronary blood vessel perfusion regarding the necessity of thorough blood filtration in perfusion experiments. It is felt that we paid insufficient attention to these warnings, but at the same time we would add that, although blood filtration through a coarse muslin filter followed by passage through a  $100 \times 200 \mu$  mesh cambric is sufficient to enable the heart-lung preparation to perform work for some hours, such filtration is not sufficient for the isolated heart preparation.

#### *Isolated heart performing work.*

Having recognized the importance of good blood filtration we returned to the experiments on the isolated heart preparation performing work. The apparatus already described in detail and the  $50 \times 100 \mu$  cambric filters were used. The first two experiments in which operations apparently went without a hitch lasted 3 and  $1\frac{1}{2}$  hours after switching over to the isolated heart from the heart-lung preparation. In spite of the fact that one of the preparations lasted 3 hours we were not satisfied with this result. A third (Fig. 7) and fourth experiment, however, lasted 6 and  $6\frac{1}{2}$  hours from the

start of the heart-lung preparation, the heart isolated from the lungs performing work for 5 and  $4\frac{1}{2}$  hours respectively; the latter experiment was terminated while the heart was still in good condition. There were slight differences in the technique in these four experiments, which should be mentioned because the third and fourth preparations were in a far better physiological condition than the first and second. Chloralose injected intravenously preceded by chloroform and ether induction was

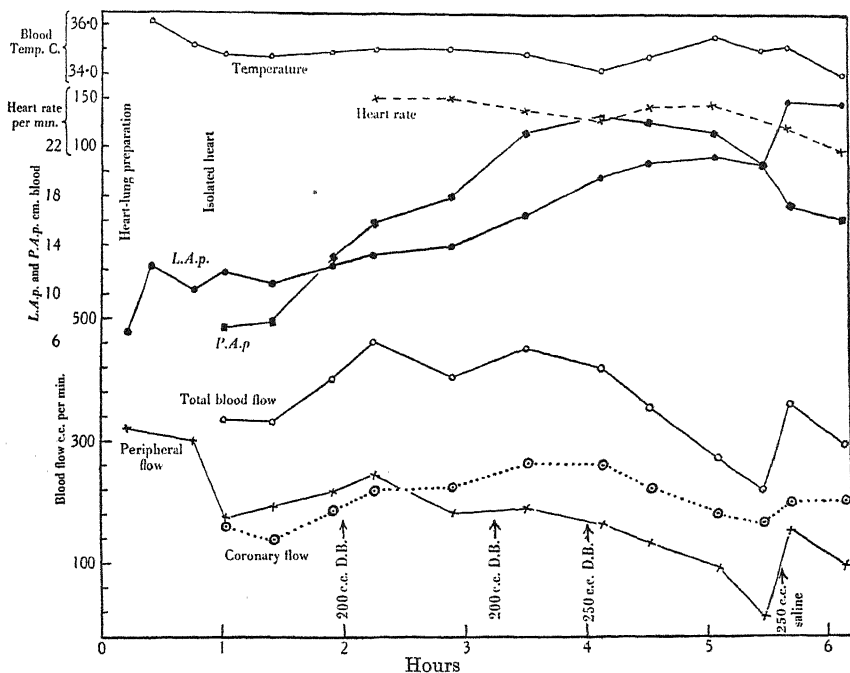


Fig. 7. Dog. 13.0 kg. Isolated heart-oxygenator preparation. Pericardium closed. B.P. = 107–111 mm. Hg. L.A.p. = left auricular pressure. P.A.p. = pulmonary arterial pressure. D.B. = defibrinated blood.

given to the preparation animals of the first and second experiments, and the extra blood was collected from animals under chloroform and ether anaesthesia. Nembutal injected intravenously under local anaesthesia was administered to all the animals used for the third and fourth experiments in the hope that the absence of chloroform during induction would improve the condition of the heart preparation. In the first three experiments defibrinated blood from the thermostat was added from time to time to the reservoir to make up any volume loss due to water evaporation

or to slight leakages, and in these experiments it was noticed that the cambric filters became clogged with a gelatinous blood film and had to be changed from three to five times in the course of the experiment. The film sometimes contained small lumps of what at first sight appeared to be fine blood clots, but on examination were found to be friable aggregations of material somewhat similar to that forming the film; that the concentrated nature of the blood plays some part in the formation of this film is not improbable. In the fourth experiment we added all the available blood to the reservoir before removing the lungs from the heart-lung preparation, *i.e.* before beginning the second stage of the operation. It may be significant that in this preparation little or no blocking of the filters took place, one filter in each position being sufficient for the whole experiment.

*Oxygen consumption.* In two experiments the oxygen consumption of the heart was measured by the method of Barcroft and Dixon [1907]. Blood samples from the reservoir, the blood first being stirred, and from the outflow tube (*f*) were taken either immediately before or after a measurement of the coronary blood flow. The blood samples (2.0 c.c.) were analysed for oxygen in Haldane's blood gas analysis apparatus by Dr J. L. Berry and Dr R. G. MacGregor to whom we wish to express our thanks; the analyses were carried out on the same day as the experiment. The results are incorporated in the Table. The oxygen capacity of the

TABLE. Oxygen consumption of isolated heart performing work in the absence of the lungs.

Exp.	Time from start hr. min.	Dog wt. kg.	Heart wt. g.		Output L./hour		B.P. mm. Hg	P.A.p. mm. Hg	Total work kg.m./ hour
			dry	wet	L.V.	R.V.			
1	5	13.0	33	187	20.3	9.7	111	5	30.0
	4 32	—	—	—	13.1	10.1	108	15	20.5
	5 14	—	—	—	20.2	12.2	107	12	30.0
2	18	6.0	21	93	15.8	3.6	134	2	27.7
	4 18	—	—	—	7.6	4.0	130	6	13.1

The figures given below are obtained from the same two experiments at corresponding time intervals.

						O <sub>2</sub> consumption c.c.			
		Reservoir blood		Coronary blood		Coronary flow c.c./min.	Per g. ht./kg.m./hour		
		O <sub>2</sub> c.c. p.c.		O <sub>2</sub> c.c. p.c.			Per g. heart wet wt./hour	Wet wt.	Dry wt.
Exp.	Time from start hr. min.	Capa- city	Con- tent	Capa- city	Con- tent		Per hour		
1	5	19.1	16.3	19.3	13.6	162	262	1.4	0.05
	4 32	30.2	24.8	31.9	21.3	168	353	1.9	0.09
	5 14	27.1	23.8	26.5	15.3	204	1040	5.6	0.18
2	18	18.5	18.35	18.5	8.0	60	373	4.0	0.15
	4 18	27.0	25.5	27.0	17.0	66	337	3.6	0.28

P.A.p. = pulmonary arterial pressure. L.V. = left ventricle. R.V. = right ventricle.

second arterial and venous blood sample in each experiment shows a large increase which is due to blood concentration consequent upon the evaporation of water from the blood on the large surfaces of the oxygenator cylinders. In the first experiment a colorimetric determination of the hæmoglobin percentage of a blood sample taken 4 hours 32 min. from the start gave a value of 160. The total amount of circulating blood at this time was 1100 c.c. and the amount at the beginning of the experiment 1850 c.c., the quantity lost therefore being 750 c.c. in 4.5 hours or 167 c.c. per hour. From these figures the calculated hæmoglobin percentage is 168, which is in fair agreement with the value obtained from the colorimetric determination. Such a rapid rate of water loss from the blood is a serious disadvantage of the oxygenator, and no doubt a closed system apparatus similar to that designed by Bayliss, Fee and Ogden [1928] would overcome this difficulty. Early on in the investigation we attempted to use two of their oxygenators in parallel but were not fortunate enough to get them working properly without "spating" at varying blood flows, although at constant blood flows we found them satisfactory. Nine minutes after the second reading of the first experiment (Table) 250 c.c. of normal saline were added to the reservoir blood, and this probably accounts for the lowered oxygen capacity of the blood at 5 hours 14 min.

The oxygen consumption of the isolated heart in the two experiments varied from 1.4 to 5.6 c.c. oxygen/g. heart/hour. These values do not differ widely from those of Evans [1912] who obtained corresponding figures for the heart-lung preparation of 3.5-6.0 and for the isolated lungs of approximately 0.9 c.c./g. heart/hour; by subtraction the intake of the isolated heart being 2.6 to 5.1 c.c. oxygen/g. heart/hour. Details of these two experiments are given in the Table, from which it will be seen that the oxygen utilization per kg.m. of work increases towards the end of an experiment of approximately 4 hours. In view of the fact that we have only undertaken the measurement in two experiments and did not adopt a method of integration, we do not wish to stress this point.

In the perfused heart Barcroft and Dixon [1907] give values from 0.6 to 5.0 c.c. oxygen/g. heart/hour, McGinty and Miller [1933] 2.4 to 5.7 and Eichholtz and Hilton [1925] 2.2 to 6.0. In the heart-lung preparation Evans and Starling [1913], using two different methods, give figures varying from 1.1 to 4.3. In the de Barenne [1919, 1921] modification of the heart-lung preparation Satani [1931] found an intake of 2.0 to 9.8 and Yoshioka [1932] of 1.6 to 6.2. In this connection Evans [1914] and Evans and Matsuoka [1915] have shown that the oxygen intake of the isolated heart is not only determined by the quantity but also by the quality of work performed.

In the isolated perfused heart the organ is doing little or no external work, and in the de Barenne preparation only the left side of the heart performs work. In our own prepara-

tion, as we have used it, the left side of the heart is delivering blood at the normal blood-pressure, whereas the right side only puts out the coronary blood flow at less than the normal pulmonary arterial pressure; finally, in the Starling heart-lung preparation and the heart *in situ* both sides of the heart carry out the normal amount of work.

In order to be able to compare the oxygen consumption of different heart preparations we have calculated the oxygen consumption in c.c./g. heart dry wt./kg.m./hour. At the end of a long experiment the heart capillaries are congested with blood and the muscle may be œdematous with a consequent increase in its weight, and this may be a source of error when comparing the weights of different hearts. To obtain the dry weight the aorta and pulmonary artery are removed close to their origin from the ventricles and the heart cavities are freed from blood by washing after opening all chambers of the heart. The organ is freed from water by draining and weighed, giving the wet weight, and then minced and dried to constant weight at 100° C. In two normal animals the ratio of dry to wet weight of the hearts was 1 : 3.5 and 1 : 4.0, and the hearts of Exps. 1 and 2 (Table I) 1 : 5.7 and 1 : 4.4 respectively. The external work of the heart was calculated by the formula of Evans [1918] although the velocity factor is hardly significant in our experiments.

*The coronary blood flow.* When the screw clip *b* is closed and the reservoir blood feeds the heart through the left auricle only, the total coronary blood is measured from the side tube *f*. Anrep [1926] considers 40–60 c.c./g. heart/hour to represent the average magnitude of the coronary circulation at a blood-pressure of 100–110 mm. Hg. The blood-pressure in some of our experiments has been somewhat higher than this, but the coronary flow has been within or only just outside the limits given by Anrep.

In two experiments we determined the influence of the addition of “fresh” defibrinated blood to the reservoir. By “fresh” defibrinated we mean blood which had been incubated at 37–39° C. for 3–7 hours and had not circulated through the heart-lung preparation: Eichholtz and Hilton [1925] used the term in this sense. No obvious permanent effect on the coronary circulation was observed (Fig. 7), although a transient one might not have been revealed by our methods of measurement. This confirms the findings of Eichholtz and Hilton on the heart-lung preparation; these workers replaced nearly all the reservoir blood with “fresh” defibrinated blood, whereas we added 200–250 c.c. of “fresh” blood to 500–1000 c.c. of circulating blood so that the “fresh” blood concentration in our experiments was smaller than in theirs.

*Heart volume.* In the heart preparations which lasted for longer than 4 hours it was surprising to find that the heart showed no dilatation



except quite at the end of the experiment. In the two experiments given in the Table, the pericardium remained intact except for a slit to allow of the insertion of the left auricular cannula, yet after 4 hours in each experiment the pericardium appeared to be quite slack and the heart small and hard. In view of the loss of efficiency at this stage as compared with the beginning of the experiment it was expected that the heart would be dilated. Since pure oxygen was passed through the oxygenator the small size of the heart was probably due to the acapnic condition of the blood [Jerusalem and Starling, 1910]. Insulin, which abolishes the progressive dilatation of the heart in the heart-lung preparation [Müller and Visscher, 1927], and glucose which has the same effect [Bayliss, Müller and Starling, 1928] were not added to the blood. At first sight it would appear that the increased oxygen consumption of the heart (Table) without an increase in diastolic volume is against the findings of Starling and Visscher [1927] and Fee and Hemingway [1927]. But Starling and Visscher expressly state that the oxygen consumption of the isolated heart (*i.e.* heart-lung preparation) maintained under constant chemical and temperature conditions is determined by its diastolic volume. They were able to maintain constant the chemical conditions during the relatively short period of time taken by their observations, whereas in our long experiments, the chemical condition of the blood at the end of the experiment probably differs markedly from that at the beginning. The absence of the lungs may possibly play some part in the phenomenon.

#### DISCUSSION.

During the investigation we had to perform a number of control experiments in order to eliminate various possible causes of early cardiac failure. These experiments showed that the following factors produced no detectable alteration in the performance of the isolated heart beating on well-filtered blood over a period of 5 hours as compared with that of the heart-lung preparation: (*a*) the removal of any nervous control arising in the lungs, (*b*) the absence of substances formed by the lungs and necessary for the normal contractile processes of the heart, and (*c*) loss of water from the blood due to evaporation in the oxygenator [Daly and Thorpe, 1933].

Our experiments, however, indicated the necessity for good blood filtration and we attach importance to two main findings, the first, that an isolated heart preparation, performing work and running on "fresh" defibrinated blood added to the reservoir after the preparation is made,

has not lasted longer than 1 hour unless the blood was well filtered before and during the experiment, and the second, that in all such successful preparations the cambric filters became clogged with a gelatinous film. We find, therefore, that fine cambric filters are as effective as the lungs in preventing the early onset of cardiac failure in heart preparations performing work and running on "fresh" defibrinated blood. To account for this phenomenon two hypotheses are put forward. The first, that the cambric filters in the heart-oxygenator preparation and the lungs in the heart-lung preparation are acting as simple mechanical filters of normal or abnormal blood constituents, which if allowed to enter the circulation gradually lead to occlusion of the coronary blood vessels. By abnormal constituents we mean those formed by changes in the defibrinated blood giving rise to a gelatinous material containing very small clots, fine shreds of fibrin or masses of blood corpuscles clumped together. The second hypothesis assumes the presence in the blood of substances responsible for the early onset of cardiac failure which are formed when the blood is defibrinated or when in the thermostat or in the artificial circulation, these being removed by the lungs in the heart-lung preparation and by the cambric filters, *e.g.* by adsorption, in the heart-oxygenator preparation. In this connection, the work of Zipf [1931] and others has demonstrated such a possibility. Our experiments do not enable us to decide whether one or both of these hypotheses are correct; both appear to satisfy the experimental findings. It may be that the postulated blood constituents or the substances of the second hypothesis are only formed in the oxygenator. Nevertheless, the lungs seem able to deal with such substances since in a heart-lung-oxygenator preparation without special filtration there was no early onset of cardiac failure.

The premiss that the lungs or cambric filters may remove substances from the blood which are responsible for the appearance of early cardiac failure leads to a speculation as to their nature, and attention was naturally attracted to the possibility that the vaso-tonins were responsible. It has been shown by Eichholtz and Verney [1924], Hemingway [1931] and Bayliss and Ogden [1933] that these substances cause constriction of the vascular system in the perfused kidney. Eichholtz and Hilton [1925], however, could obtain no evidence that "fresh" defibrinated blood had any effect on the coronary flow in the heart-lung preparation; in their experiments the "fresh" blood had to pass once through the lungs before reaching the coronary vessels and there is the possibility that the vaso-tonins might have been destroyed even in this short time [see Eichholtz and Verney, 1924]. As mentioned previously

our own observations confirm those of Eichholtz and Hilton in the heart-oxygenator preparation with blood filtered through fine cambric. It would appear, therefore, that if the early onset of cardiac failure in the heart-oxygenator preparation running on poorly filtered blood is due to the presence of vaso-tonins, they are removed by the insertion of fine cambric filters. For perfusion of the coronary vessels, Anrep and Häusler [1928] found it necessary to circulate the blood in the heart-lung preparation for about half an hour, otherwise the defibrinated blood caused vigorous and protracted vaso-constriction. In all our experiments, except one, only about half the total blood finally used circulated through the heart-lung preparation before switching over to the isolated heart circuit, the other half was then added to the reservoir. Under these conditions we observed no significant coronary vaso-constriction (Fig. 7) provided fine cambric filters were used. In the excepted experiment all the blood eventually used for the heart-oxygenator preparation was circulated through the heart-lung preparation and in this case little or no clogging of the filters occurred, but we hesitate to lay stress upon this observation in a single experiment. It is not improbable that the formation of the gelatinous film on the filters may in part be determined by the biological properties of the bloods obtained from the two or three animals used and their effect upon one another when mixed together. It may be said that our observations taken in conjunction with those of Anrep and Häusler suggest that the insertion of cambric filters in the isolated heart preparation, to which "fresh" defibrinated blood is added from time to time, obviates the necessity of circulating the "fresh" blood through a heart-lung preparation.

It is interesting to note that the insertion of a fine cambric filter in a pump-oxygenator-kidney circuit does not result in an elevation of the blood flow to the level usually found in pump-lung-kidney preparations [Bayliss and Ogden, 1933], the presumption being that the filter does not remove the vaso-tonins. This observation suggests that the vaso-tonins which act upon renal blood vessels may be circulating in the blood of our heart-oxygenator preparations and that if there is any truth in our second hypothesis, the renal vaso-tonins and the substances responsible for early cardiac failure are not identical.

The term "detoxicated" defibrinated blood has been used for blood which has been passed through the lungs and thereby lost its vaso-constrictor properties, and we have seen there is abundant evidence that such a process occurs when the blood is tested on the isolated perfused kidney. That this mechanism of "detoxication" may prevent the onset

of early cardiac failure in the heart-oxygenator preparation is not denied but it has yet to be proved. Moreover, our observations raise the question as to the possibility of an additional mechanism being involved in the so-called "detoxication" of blood, namely one of mechanical filtration of particulate material in the blood.

#### SUMMARY.

1. An isolated mammalian heart-oxygenator preparation, in which the quantity and quality of work performed by each side of the heart can be varied at will, is described.

2. Early cardiac failure occurs in the heart-oxygenator preparation running on "fresh" defibrinated blood unless the blood is filtered through fine cambric filters before use and during circulation. Possible causes of this failure are discussed.

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## THE INFLUENCE OF THE PARATHYROID ON THE METABOLISM OF CREATINE AND PHOSPHORIC ACID.

BY C. G. IMRIE AND CONSTANCE N. JENKINSON.

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THERE are reasons for associating the parathyroid glands with the metabolism of creatine phosphate in the muscles. It has been observed that the rate of resynthesis of creatine phosphate after stimulation of the muscles of thyroparathyroidectomized cats which have developed tetany is very much slower than in normal ones; also that the rate of this resynthesis in thyroparathyroidectomized cats which have recovered from tetany following the administration of parathormone, is similar to that observed in normal animals [Imrie and Jenkinson, 1932]. These observations, however, have been based on a comparison of results obtained from groups of animals. They would be more convincing if the effect of the parathormone could be demonstrated in the same animal.

Collip, Clark and Scott [1925] observed that the rise in serum calcium which follows the administration of parathormone reaches a maximum in 5-9 hours. It is conceivable therefore that the influence of this hormone on the creatine phosphate might be detectable within this limit of time. Accordingly some experiments have been carried out in order to test this hypothesis. In these, the rate of resynthesis of creatine phosphate after stimulation of the muscles was observed in one limb of a cat and several hours later similar observations were made on another limb. In some instances parathormone was given intravenously immediately after the muscles of the first limb were removed for analysis. If the hormone exerted any influence on the creatine phosphate within the time limits of the experiment, it should be apparent in the values obtained in the muscles of the second limb studied several hours later.

### EXPERIMENTAL PROCEDURE.

Young cats were used. Thyroparathyroidectomy was performed under ether anaesthesia. The animals were permitted to recover and, after the symptoms of tetany were well-marked, the experiments were carried out.

Anæsthesia was induced with ether and maintained with chloralose. Immediately after the induction of anæsthesia the crural and sciatic nerves of one hindlimb were cut and the animal left for an hour to recover from the effect of the ether and from changes in the creatine phosphate which might arise from the muscular movements associated with the induction of anæsthesia. A portion of muscle was removed for examination and then the peripheral ends of the divided nerves were faradized for 10 min. The two electrodes employed were attached to the same induction coil. Analyses of the muscles were made at 15 or 20 min. intervals for an hour. Several hours later this procedure was repeated on the other hindlimb. The animals were kept under the influence of chloralose throughout the whole period. In some instances intravenous injections of parathormone were given immediately after the examination of the first limb was completed, in others no parathormone was given. The Eggletons' method [1929] for the phosphates was followed throughout except that the muscles were immersed in liquid air immediately after removal. It was found that the cat's muscle pulverized more readily when this procedure was adopted.

### RESULTS.

In Table I are set out figures for the creatine phosphate in the muscles of two thyroparathyroidectomized cats before and after stimulation. The corresponding graphs are shown in Fig. 1. The rates of resynthesis of

TABLE I. Creatine phosphate in the muscles of the hindlimbs of thyroparathyroidectomized cats before and after stimulation. The muscles of one limb were examined several hours after the other. Figures express creatine phosphate as mg. phosphorus per 100 g. muscle.

	Before stimu- lation	Zero	15 min.	30 min.	45 min.	60 min.
<i>Exp. 1</i>						
1st limb	42.5	19.9	22.2	25.0	28.9	33.9
2nd limb	40.6	17.6	20.0	22.2	25.8	28.9
6 hours later						
<i>Exp. 2</i>			20 min.	40 min.	60 min.	
1st limb	43.5	17.5	21.6	23.6	27.5	
2nd limb	45.4	16.9	20.4	22.3	26.6	
4 hours later						

creatine phosphate are strikingly similar in the separate limbs of each animal. The initial values for the creatine phosphate are lower and the rate of resynthesis after stimulation is much slower than that observed in normal cats [Imrie and Jenkinson, 1932].

If, however, parathormone is injected intravenously immediately after the muscles of the first limb have been removed for examination and observations on the second limb are made 4-7 hours later, the rate of resynthesis of the creatine phosphate after stimulation is more rapid than it was in the first limb where no parathormone had been given. The

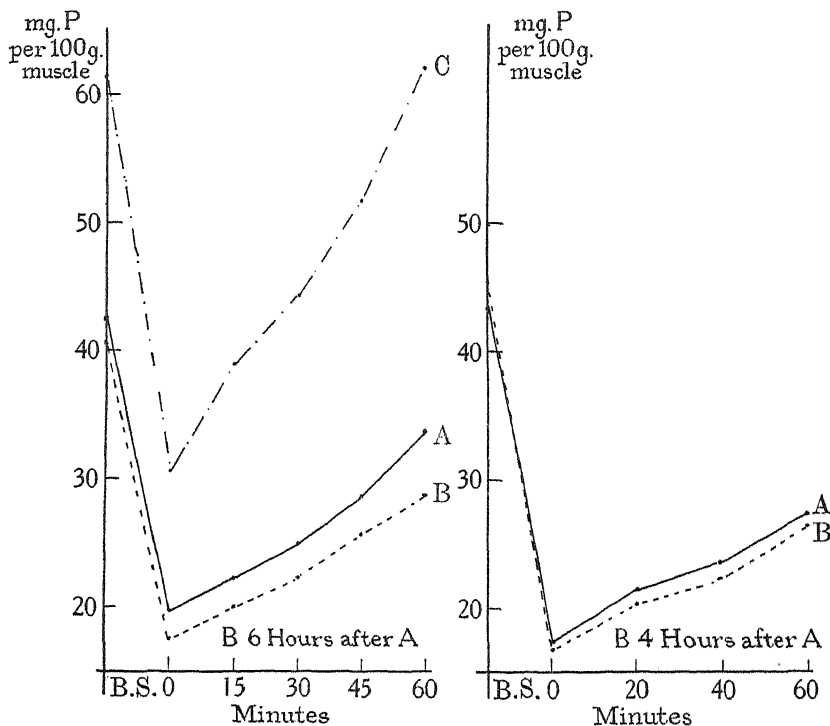


Fig. 1. Creatine phosphate in the muscles of thyroparathyroidectomized cats before and after stimulation. *A*, right hindlimb; *B*, left hindlimb; *C*, creatine phosphate in the muscles of a normal cat, similarly stimulated. Ordinates, mg. phosphorus in the form of creatine phosphate per 100 g. muscle; abscissae, time: B.S., before stimulation; 0, immediately after stimulation.

results of four such experiments are included in Table II, and for one experiment are expressed graphically in Fig. 2. In each instance, though the initial values for the creatine phosphate have remained unaltered, the rate of the resynthesis of the creatine phosphate is more rapid after the parathormone has been given; in fact it resembles that observed in normal animals.

On two occasions, Exps. 7 and 8, creatine in amounts corresponding



to 200 mg. per kg. body weight was injected intravenously as well as the parathormone, after the muscles of the first limb had been removed for examination. The results are given in Table II.

TABLE II. Creatine phosphate in the muscles of the hindlimbs of thyroparathyroidectomized cats before and after stimulation. Immediately after the muscles of one limb were removed for examination 10 units of parathormone were injected intravenously and several hours later the muscles of the other limb were studied. In Exps. 7 and 8 creatine in amounts corresponding to 200 mg. per kg. body weight were injected along with the parathormone. Figures express creatine phosphate as mg. phosphorus per 100 g. muscle.

Exp. No.		Before stimulation	Zero	15 min.	30 min.	45 min.	60 min.
3	1st limb	53.8	29.4	32.7	37.0	37.6	38.8
	2nd limb	55.5	25.5	34.4	40.6	42.4	50.5
	6½ hours later						
4	1st limb	47.8	25.0	31.2	32.1	34.4	38.6
	2nd limb	49.0	24.5	36.1	39.0	43.4	47.5
	7 hours later						
				20 min.	40 min.	60 min.	
5	1st limb	47.1	19.4	24.4	25.2	35.4	
	2nd limb	51.5	17.6	37.0	37.6	49.4	
	5½ hours later						
6	1st limb	49.8	18.8	21.6	27.6	28.0	
	2nd limb	50.2	18.8	27.4	34.4	45.5	
	5 hours later						
7	1st limb	43.2	21.2	24.4	32.3	35.5	
	2nd limb	46.8	21.5	32.7	37.2	50.0	
	5 hours later						
8	1st limb	54.6	30.3	40.4	47.3	53.5	
	2nd limb	60.0	32.2	39.1	58.4	63.0	
	5½ hours later						

It is to be seen that the initial values for the creatine phosphate are slightly higher in the second limb than in the first. The curves of resynthesis of the creatine phosphate are similar to those obtained in cats which had received parathormone alone, except that in both instances the concentration of creatine phosphate in the muscles removed at the end of the hour was higher than it was before the muscles were stimulated. In Exp. 8 it is to be noted that the values for the creatine phosphate before and after stimulation of the muscles of the first limb are practically normal. This cat had its thyroids and parathyroids removed 2 days previously, but tetany was not well-marked and there was no dyspnoea at the time the experiment was begun. Nevertheless, the difference in the rate of resynthesis of the creatine phosphate after creatine and parathormone had been given is quite marked, and the final value for the creatine phosphate was higher than the initial one.

A few experiments of a different nature have been carried out in order to observe the creatine phosphate before and after stimulation in the muscles of normal cats which have been given creatine in the food with or without injections of parathormone for several days before the

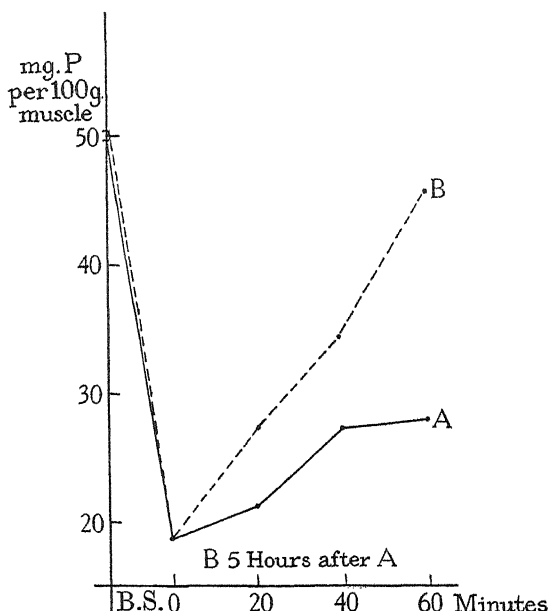


Fig. 2. Creatine phosphate in the muscles of thyroparathyroidectomized cats before and after stimulation. Parathormone injected intravenously after the muscles of the first hindlimb were removed for examination: the muscles of the second hindlimb were studied several hours later. *A*, right hindlimb before parathormone; *B*, left hindlimb after parathormone. Ordinates, mg. phosphorus as creatine phosphate per 100 g. muscle; abscissæ, time: B.S., before stimulation; 0, immediately after stimulation.

TABLE III. Creatine phosphate in the muscles of normal cats before and after stimulation.

In Exps. 9 and 10, 5 g. of creatine were given daily for several days before the observations were made. In Exps. 11, 12 and 13, in addition to the creatine, parathormone in doses of 10 units was given subcutaneously as indicated. Figures express creatine phosphate as mg. phosphorus per 100 g. muscle.

Exp. No.	Days of treatment	Before stimulation	Zero	20 min.	40 min.	60 min.
9	9	62.0	17.7	38.3	46.7	62.4
10	7	59.5	17.5	28.9	43.0	58.1
11	9	68.2	22.0	34.8	40.8	55.4
12	8	69.0	17.3	33.4	49.4	52.0
13	3	*A 72.0	39.2	50.0	59.4	55.0
		*B 71.4	35.3	49.7	58.5	57.5

\* In this experiment the muscles of both hindlimbs were examined, *B* 4½ hours after *A*.

observations were made. It is known that the creatine content of cat's muscle may be increased by feeding creatine [Folin and Denis, 1912], and that the creatine phosphate may also be increased when creatine is injected into cats which have received parathormone previously. In

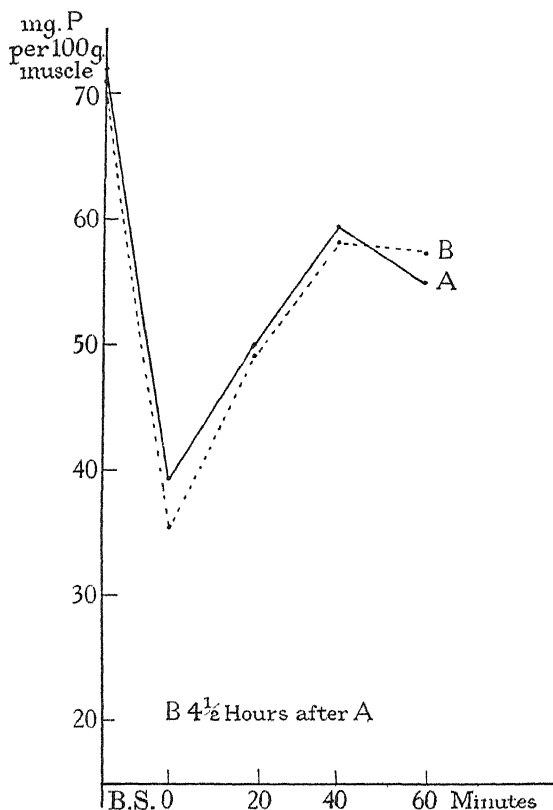


Fig. 3. Creatine phosphate in the muscles of a normal cat which had received 5 g. creatine + 10 units of parathormone for 3 days. *A*, right limb; *B*, left limb  $4\frac{1}{2}$  hours later. Ordinates, mg. phosphorus in the form of creatine phosphate per 100 g. muscle; abscissæ, time: B.S., before stimulation; 0, immediately after stimulation.

Table III are set out the results obtained in two experiments, Nos. 9 and 10, where creatine alone was given in daily doses of 5 g. for 7 and 9 days respectively. The initial values for the creatine phosphate and the rate of its resynthesis after stimulation of the muscle correspond to the average values shown in our former paper.

In three instances (Exps. 11, 12 and 13), where creatine in similar amounts was given along with daily doses of 10 units of parathormone, the initial values for the creatine phosphate are above the normal in every instance. After stimulation the creatine phosphate falls as in the normal animal and does not return to the original high level within the hour but reaches a value similar to that observed in the normal untreated animal. In one experiment, No. 13, where both creatine and parathormone were given, the muscles of one limb were examined  $4\frac{1}{2}$  hours after the other. The values for the creatine phosphate follow each other closely. The initial values are higher than the normal average: the values found immediately after stimulation are not so low as in the other experiments and at the end of an hour they have not returned to the original high level, but as in Exps. 11 and 12 reach a figure observed in the normal untreated animal.

In all the experiments described in this paper the orthophosphates as well as the other forms of phosphorus were determined. The orthophosphates showed reciprocal changes to the creatine phosphate. This was observed in other experiments described in a former paper.

#### CONCLUSIONS.

The results of the experiments described in this paper confirm those which have been published previously. They show that in thyroparathyroidectomized cats the concentration of creatine phosphate in the muscles is lower than in normal ones; also that the rate of resynthesis of creatine phosphate following stimulation of the muscles is much slower in the former than in the latter animals. They also show quite clearly that following the administration of parathormone to thyroparathyroidectomized cats the resynthesis of creatine phosphate after its concentration in the muscle has been lowered by stimulation takes place at a rate similar to that observed in normal animals. This has been demonstrated in the same animal in the course of individual experiments and is therefore more convincing than the results published previously which were obtained in groups of animals. It has also been shown that this effect of the parathormone is manifest within at least 5 hours of its administration intravenously.

Where creatine is administered along with parathormone in feeding experiments for some days or in acute experiments there appears to be a definite effect upon the initial values of the creatine phosphate in the muscles and the rate of its resynthesis after stimulation.

It is a pleasure to acknowledge our thanks to Prof. Leathes for his interest and advice.

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## THE EXCRETION OF PROLAN AFTER INTRAVENOUS INJECTION INTO THE RABBIT.

By A. S. PARKES

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AND W. E. WHITE

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### I. INTRODUCTION.

THE abundance of ovary-stimulating substance ("Prolan") in the urine of pregnant women suggests that it is easily excreted, and also, unless it is present in the circulation in enormous quantities, that an appreciable proportion may be removed in this way. By contrast, the apparent absence of the substance from the urine of most lower mammals at all times of the oestrous cycle is remarkable, but little experimental work seems to have been carried out on the possibilities of excretion of the hormone by animals such as the rabbit. Smith and Moore [1933] were unable to demonstrate that any appreciable amount of the thyroid-stimulating hormone present in anterior lobe extract was excreted by guinea-pigs after subcutaneous injection. Snyder and Wislocki [1931] injected three rabbits with human urine of pregnancy, and subsequently tested the rabbit urine on other rabbits. Ovulation was obtained in one test animal. The present paper deals with more extensive experiments on these lines, carried out by a different technique and with a prolan extract of known potency.

### II. TECHNIQUE.

*Prolan preparation.* The same preparation of prolan was used throughout the experiments. It was stored as a dry powder at about  $-2^{\circ}\text{C}.$ , other experiments having shown it to be indefinitely stable under these conditions. Appropriate amounts of the powder were made up into fresh aqueous solutions for each experiment. The potency of the extract had previously been determined by injection into groups of at least 20 normal oestrous rabbits. 10 mg. per rabbit caused ovulation in 98 p.c., while

5 mg. gave the effect in just over 50 p.c. 10 mg. has therefore been considered as the amount required to cause ovulation with comparative certainty.

*Collection of urine.* Since the time over which the urine had to be collected was comparatively short, it was possible to avoid the use of metabolism cages and thus to obtain the urine fresh and uncontaminated. Our technique was to open the abdomen under anaesthesia, empty the bladder, ligate the urethra, inject intravenously and, after the required time, to kill the animal, remove the bladder whole and drain into a measuring cylinder. Males and females were used for the excretion tests, but all animals were gonadectomized before injection.

*Preparation of rabbit urine for injection.* Since we aimed at using the whole of the urine collected from each injected rabbit some process of purification was necessary, but at the same time it was highly desirable to avoid loss of the hormone. Our technique was therefore to make up the urine to 80 p.c. alcohol by the addition of absolute alcohol, and to centrifuge off the precipitate and take it up in water. Even so, the resulting extract was found to be highly toxic unless other precautions were observed. All the urine extracts made from three rabbits injected soon after feeding killed the respective test animals. On the other hand, to secure adequate excretion during the experiment, it was necessary to give water after injection if food had previously been withheld<sup>1</sup>. One preparation made from a rabbit given only water on the day of the experiment was tolerated easily by the test animal. To avoid giving water to drink and to standardize the intake, our regular technique has been to give 100 c.c. saline intravenously at the time when the prolan was injected. By this means the total volume of urine excreted by the rabbit during the experiment, though very variable (20–80 c.c.), was kept about normal. We have no reason to suppose that the injection of saline altered the conditions of excretion of the hormone, and the one rabbit not given saline produced active urine.

*Test animals.* The rabbit urine extracts were tested by injection into oestrous rabbits; laparotomy was performed next day to ascertain whether ovulation had been induced.

### III. EXPERIMENTAL RESULTS.

The amounts of prolan given to the rabbits were 30 mg. (3 exps.), 50 mg. (4 exps.) and 100 mg. (7 exps.), or about three, five and ten times the amount required to produce ovulation with certainty in an oestrous

<sup>1</sup> With a large ration of green stuff, water is not necessary in keeping rabbits.

animal. After prolan injection, urine was collected for times up to 9 hours, two samples (0-3 and 3-9 hours) being taken from one animal (EP 11). Even with the precautions mentioned above, collection for more than 9 hours produced highly toxic samples. The experiments are summarized in Table I, which also gives the results of the tests on the rabbit urine.

TABLE I. Excretion of prolan by the rabbit.

No. of exp.	Amount of prolan powder given (mg.)	Time urine collected after in- jection (hours)	Proportion of urine extract given to test animal	Result of test
EP 1	30	7	All	Negative
16	30	9	All	Positive
17	30	9	All	Negative
2	50	7	All	Negative
12	50	6	All	Negative
13	50	9	All	Positive
21	50	9	All	Positive
3	100	5	All	Positive
4	100	5	All	Negative
6	100	5½	All	Positive
11	100	3	All	Positive
11	100	3-9	All	Positive
5	100	9	All	Positive
18	100	9	1/4	Positive
18	100	9	1/2	Positive
15	100	9	1/4	Negative

These results suggest the following considerations:

(a) Of two animals receiving 30 mg. (*i.e.* three ovulation doses), one gave active urine 9 hours later, so that rather less than one-third of the hormone appears to have been excreted during this time and recovered.

(b) Of the two tests on one-quarter of the urine obtained 9 hours after the injection of 100 mg. (*i.e.* ten ovulation doses), one was positive and the other negative. In other experiments, injection of all the urine and also of one-half of the urine collected 9 hours after injection of 100 mg. produced positive reactions.

These results again suggest that about one-third or less of the material is excreted in 9 hours. It would thus appear that the blood threshold, if any, which the hormone must attain before excretion can begin is comparatively low.

(c) The results suggest, though they do not prove, that excretion continues up to 9 hours. Thus, in both the 30 mg. and 50 mg. group, the urine appears to be more active after 9 hours than after 6 or 7 hours. Further, after the administration of 100 mg., active urine was obtained during the first 3 hours and also between 3 and 9 hours.



We have not determined what happens to the two-thirds of the hormone unaccounted for in the above results. Presumably some destruction takes place in the blood, and also excretion may well continue after 9 hours. Our results, however, do show that, given enough prolان in the blood, the kidneys of the rabbit can excrete sufficient in under 3 hours to cause ovulation in an oestrous rabbit, *i.e.* about 4 units per day per kg. body weight.

A pregnant woman excretes 500-1000 rabbit units of prolان per day, or roughly 10 units per day per kg. The amount which can be excreted by a rabbit is therefore of the same order as that which actually is excreted by a woman. It may thus be concluded that the failure of the pregnant rabbit to excrete prolان is due not to inability on the part of the kidneys, but more probably to lack of the hormone in the blood.

#### IV. SUMMARY.

1. Prolان was injected intravenously into gonadectomized rabbits. About one-third of the hormone given was recovered from the urine within 9 hours. The amount excreted by these rabbits was of the same order (per kg. body weight) as the output of the pregnant woman.

2. The kidneys of the rabbit are, therefore, able to excrete appreciable quantities of the hormone, and their failure to do so during pregnancy is presumably due to lack of the substance in the blood.

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## THE EXTRA-UTERINE SURVIVAL OF SPERMATOZOA.

BY WILLIAM E. WHITE<sup>1</sup>.*(From the National Institute for Medical Research, London.)*

WHILE spermatozoa live in the epididymis of the male rat for about 42 days [Moore, 1928; White, 1932], their life is a matter of hours when they are introduced into the female genital tract. There are two main factors causing this shortened life—the violent activity which sperm display when dispersed in the accessory secretions of the male and the increased temperature to which they are subjected in the female. The question arises as to whether the uterus is the most favourable environment in the female for sperm survival or whether in addition to the two first mentioned factors the uterus itself exerts deleterious effects. Although Kugota [1929] reported that the uterine secretions of the rat during oestrus had a beneficial effect on sperm survival *in vitro*, to test this point adequately it is necessary to confine sperm in another cavity where they may be subjected to conditions approximating to those in the uterus. The abdominal cavity is unfavourable [Hoehne and Behne, 1913]. Accordingly sperm were injected into the lateral ventricle of the brain. Here they are freely motile, are exposed to the same temperatures as in the uterus, and may readily be recovered for examination. Data on the survival of sperm in this environment will be given in the present paper.

## MATERIAL AND METHODS.

Thirty-four female rats in oestrus or pro-oestrus and twenty-one males were used in this work. The technique for injecting the sperm into the lateral ventricle was briefly as follows: Under ether anaesthesia a median incision was made in the skin over the calvarium. The fascia was removed from a small area over the parietal bone and a hole 1 mm. in diameter trephined approximately 3 mm. posterior to the fronto-parietal suture and 2 mm. lateral to the sagittal suture. Sperm were removed from the tail of the epididymis or ductus deferens and mixed with a small quantity of

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Baker's solution or Tyrode's solution. With the aid of a very fine flatly ground hypodermic needle 0.02–0.03 c.c. of this sperm suspension were injected into the lateral ventricle through the hole in the skull. No bleeding usually resulted. This seems to be an important point, since in the few cases where slight hæmorrhage occurred negative results were obtained. It is necessary to direct the needle at the proper angle and only to the correct depth in order to penetrate the ventricle, which is little more than a potential cavity.

If more than 0.05 c.c. of solution is injected into the ventricle the animals sometimes show symptoms of cerebral pressure after coming out of the anæsthetic. For this reason only about half this quantity, which is sufficient for recovering large numbers of spermatozoa, was injected. After the appropriate interval the animals were killed by decapitation and the condition of the sperm determined by laying the ventricle open and removing the contents with a small pipette for microscopical examination.

For the injection of sperm into the uterus Yochem's [1929] technique, which consisted of ligating the uterus above the cervix and injecting the sperm suspension into both horns, was followed. Although he injected  $\frac{1}{2}$  c.c. into each horn, 0.1 c.c. seems to contain ample sperm and does not put the œstrous uterus, already distended with fluid, under undue pressure.

Moore's [1928] method for quantitatively estimating the degree of motility was used. Maximum motility by this system is \*\*\*\*, meaning that all sperm are violently motile. When all but a few sperm are considerably motile the designation is \*\*\*. Likewise when only half are somewhat motile the reading is \*\*, and when only a few are still motile, even one or two, the designation is \*. Total absence of motility is 0. Normal sperm from the tail of the epididymis or ductus deferens always show \*\*\*\* motility when mixed with Tyrode's or other physiological solutions.

## RESULTS.

Motility has been detected in sperm from the lateral ventricle of the brain on one occasion  $17\frac{1}{2}$  hours after injection and frequently after 15 hours (Table I). Sperm from the tail of the epididymis seemed to survive longer than those from the ductus deferens, which is also the case in the male after epididymal isolation [see White, 1932]. Since the animals had not copulated for some time before the experiments were begun, they undoubtedly had older and less vigorous sperm in the distal

TABLE I. The survival of spermatozoa in the lateral ventricle of the rat brain.

Animal	Time after injection hours	Sperm taken from	Motility	Number of sperm recovered
B 1	4 $\frac{1}{2}$	Ductus deferens	0	Very few
BH 2	4 $\frac{1}{2}$	Tail epididymis	*	Many
BH 3	7 $\frac{1}{2}$	Ductus deferens	*	"
B 44	10	" "	0	Very few
B 61	11	" "	0	"
W 91	11 $\frac{1}{2}$	Tail epididymis	*	Many
B 64	12 $\frac{1}{2}$	" "	*	"
W 28	12 $\frac{1}{2}$	" "	*	"
B 53	15	Ductus deferens	0	Few
W 30	15	Tail epididymis	*	Many
G 00	15	" "	*	"
W 33	15	" "	*	"
W 12	17 $\frac{1}{2}$	" "	0	"
BH 99	17 $\frac{1}{2}$	" "	0	"
B 00	17 $\frac{1}{2}$	" "	0	"
BH 4	17 $\frac{1}{2}$	" "	*	"
W 21	17 $\frac{1}{2}$	" "	0	"
B 45	18	" "	0	"
GH 43	18	" "	0	Very few
W 4	18	" "	0	Many

end of the ductus deferens than in the epididymis. In a single case Yochem [1929] found motile sperm 12 $\frac{1}{2}$  hours after injection into the uterus of an oestrous rat. None were found after longer periods. The writer has been unable to recover motile sperm for longer than 10 hours after injection into the uterus, although a number of animals were examined (Table II).

TABLE II. Survival of spermatozoa injected into uterus of oestrous rat\*.

Animal	Time after injection hours	Motility	Number of sperm recovered
7	5	*	Few
8	5	0	"
10	7 $\frac{1}{2}$	*	Very few
9	10	0	Few
11	10	*	Many
13	10	0	"
14	10	0	Few
5	12 $\frac{1}{2}$	0	None
6	12 $\frac{1}{2}$	0	Few
1	15	0	Very few
2	15	0	Few
3	17 $\frac{1}{2}$	0	"
4	17 $\frac{1}{2}$	0	Many

\* In this series all sperm for injection were removed from the tail of the epididymis.

The conditions after the injection of sperm are possibly not altogether comparable to those after normal copulation, since the sperm were not mixed with the accessory secretions of the male. It was desired to obtain data on sperm survival after copulation, but several attempts to get

timed matings at night by observing the animals or examining the females at 15 or 30 min. intervals were unsuccessful. Yochem was more fortunate. He found motile sperm after 12 hours in the uterus, after 16 and 17 hours in the oviducts, and after 14½ hours in the vagina. This relatively long survival in the vagina was probably due to the fact that rats usually form copulation plugs. Sperm confined in such a plug are doubtless protected from the vaginal secretions which in most mammals are very unfavourable for sperm survival.

#### DISCUSSION.

Yochem [1929] found motile sperm in the Fallopian tube 17 hours after mating, but in the uterus for only 12 hours. Likewise motile sperm have been recovered from 15–17½ hours after injection into the lateral ventricle of the brain. The longer survival in the tubes than in the uterus may be due either to a more favourable environment there, or to the more vigorous sperm leaving the uterus and reaching the Fallopian tube.

The shorter survival of sperm in the uterus than in the lateral ventricle of the brain, where they are often motile for 15 hours after injection, indicates that the brain cavities are possibly more favourable for the survival of highly motile sperm. This suggests that, at least in the case of the rat, the uterus is not specifically adapted for the longest possible survival of motile sperm. The fact that the uterus is slightly less suitable may be due to the presence of leucocytes, many of which are found even in the oestrous uterus, or to the presence of unfavourable secretions.

#### SUMMARY.

A comparison of the survival of sperm in the uterus and in another body cavity, the lateral ventricle of the brain, has been made.

Sperm have survived in the brain for 17½ hours, 5 hours longer than in the uterus.

It is concluded that if the uterus is not antagonistic, it is at least not specifically favourable for the maximum survival of highly motile sperm.

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## HISTAMINE IN CANINE GASTRIC TISSUES.

By GERTRUDE GAVIN, E. W. McHENRY  
AND M. J. WILSON.

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IN 1905 and 1906 Edkins reported that aqueous extracts of pyloric mucosa, when injected subcutaneously, caused secretion of gastric juice in the dog. Extracts of fundic mucosa did not stimulate gastric secretion, although they were stated to be more potent in lowering blood-pressure than the pyloric extracts. As a result of this work, the second or chemical phase of gastric secretion is now believed by many to be due to the formation in the pyloric mucous membrane of a specific substance called "gastrin," which when absorbed into the blood stimulates the gastric glands.

In 1920 Popielski demonstrated that histamine caused gastric secretion, and since then there has been considerable speculation as to whether histamine and "gastrin" are identical. In 1932 Sacks, Ivy, Burgess and Vandolah reported the isolation of histamine from hog pyloric mucosa. They showed that, after incubation with histaminase, saline extracts of this tissue no longer produced gastric secretion. They concluded that if a "gastric hormone" has been extracted from the pyloric mucosa the active principle is histamine. These workers consider the possibility that the "gastric hormone" may not as yet have been extracted from the mucosa. In these studies no reference was made to the investigation of any part of the stomach other than the pyloric mucosa.

In view of this similarity between "gastrin" and histamine it seemed advisable to study the distribution of the amine in various parts of normal stomachs. For this purpose dogs, after 16 hours' starvation or in some cases after a meat meal, were used. The animals were killed instantly by means of a captive-bolt pistol, the stomach was removed immediately, wiped as clean as possible with dry gauze, and then dissected into pyloric mucosa and muscle, and fundic mucosa and muscle. Each section was then finely minced, and weighed portions used for histamine determina-

tions. Extracts were prepared by our customary hydrochloric acid procedure [Best and McHenry, 1930]. The minced tissue was thoroughly mixed with hydrochloric acid within 30 min. after the death of the dog, and it is unlikely that any change occurred in the histamine content of the tissue in the interval, especially since the gastric tissues of the dog do not contain histaminase in amounts detectable by the methods available [Best and McHenry, 1930]. The depressor material present was determined by assay against a standard histamine solution, using the blood-pressure method in an etherized cat. Table I shows the depressor contents calculated as milligrams of histamine per kilogram of tissue.

TABLE I. Histamine content of dogs' stomachs (mg./kg.)

No.	Condition	Pyloric		Fundic	
		Mucosa	Muscle	Mucosa	Muscle
831	24 hr. starvation	28	17	48	16
856	24    "	40	4	60	14
857	30    "	50	15	90	33
869	24    "	37	19	80	27
	Average	39	14	69	22
842	1 hr. after meat meal	54	18	72	25
858	2    "	100	50	110	60
860	1½   "	46	30	80	28
861	2    "	54	15	105	38
	Average	63	28	92	38

A number of depressor substances, in addition to histamine, may be present in these tissues. Extracts prepared by acid digestion are free from adenylic acid and related compounds, and from "callicrein," since such treatment destroys these substances. All extracts were assayed before and after atropinization of the cat. Since these two assays were always identical it may be stated that the solutions did not contain choline or acetylcholine in amounts sufficient to be detectable by this procedure. However, an etherized cat does not respond to injections of small amounts of choline, and more sensitive methods have shown this compound to be present. Assay of the solutions against acetylcholine on isolated rabbit intestine showed small amounts of choline compounds to be present, and this result was confirmed by assays after acetylation of the solutions. These assays were kindly performed for us by Mr O. M. Solandt.

Incubation of the solutions with added histaminase under standard conditions as to pH and temperature [McHenry and Gavin, 1932] caused a disappearance of all the depressor activity. It has not yet been demonstrated that this enzyme is specific for histamine, but it does not

affect several related amines and iminazole compounds or choline. Incubation without histaminase did not alter the depressor effects of the gastric extracts.

Previous work in this laboratory showed that histamine was not formed by the acid digestion procedure. Pure egg albumin and pure casein, 2 g. in each case, were carried through the process and the resultant extracts showed no depressor action. Solutions of *l*-histidine dichloride were similarly treated with the same result. After adding *l*-histidine dichloride to minced stomach, the mixture was extracted, and no increase was found in the depressor content of the final extract when compared with controls from the same lot of tissue. However, this procedure might have liberated the amine from a loosely bound complex in which it already existed in the decarboxylated form. Simple saline extracts of gastric tissues were found to contain a depressor substance which was similar to that present in the extracts resulting from acid digestion, and these saline extracts likewise lost their depressor effect when incubated with histaminase.

Although these extracts of gastric tissues contained small amounts of choline compounds, the evidence cited in the above three paragraphs justifies the conclusion that all the depressor activity measured by the etherized cat procedure was due to histamine.

Table II gives the average total amounts of histamine present in each type of tissue. Not only does fundic mucosa contain more histamine per

TABLE II. Histamine content of a fasting dog's stomach.

Tissue	Weight g.	Amount of histamine mg.	P.c. of total
Pyloric mucosa	15	0.6	12
Pyloric muscle	8	0.1	2
Fundic mucosa	60	4.1	80
Fundic muscle	15	0.3	6
Total		5.1	

kilogram than does pyloric mucosa, but there is much more of the former tissue in the stomach. A surprisingly large amount, 80 p.c. of the total histamine content of the stomach, was present in the fundic mucosa, while only 12 p.c. was found in the pyloric mucosa. If the suggestion of Sacks, Ivy, Burgess and Vandolah is correct, that "gastrin" is histamine, then the fundic mucosa is probably a much more important source than the pyloric section. Muscle from either part of the stomach contains little histamine.



Since Edkins has stated so definitely that fundic extracts do not produce secretion, and this has received general acceptance, it seemed essential to carry out experiments on this point. For this work a dog with a gastric fistula was prepared for us by Dr Best. This dog has been given a number of solutions subcutaneously, and the stomach contents have been collected at 5-min. intervals after the injection, by means of a catheter inserted through the fistula. The results of these experiments are summarized in Table III. Simple saline extracts of fundic mucosa,

TABLE III. Secretagogue effects of gastric extracts.

Injected solution		Volume of gastric juice collected in 30 min. c.c.
Volume and nature	Histamine content mg.	
0.22 c.c. histamine standard	0.2	40
0.5 c.c. histamine standard	0.5	40
50 c.c. solution prepared by HCl digestion of fundic mucosa	0.8	50
200 c.c. saline extract of fundic mucosa	0.7	50
90 c.c. saline extract of pyloric mucosa	0.2	20
70 c.c. saline extract of fundic mucosa	0.4	60
70 c.c. solution from same lot incubated with histaminase	0 (formerly 0.4)	None
70 c.c. solution from same lot incubated without histaminase	0.4	60
30 c.c. of same lot after 6 days in refrigerator	0.2	20
40 c.c. saline extract of pyloric mucosa incubated with histaminase	0 (formerly 0.2)	None
60 c.c. saline	0	None

and solutions prepared from similar tissue by acid digestion, definitely caused gastric secretion. Their power to do so was destroyed by incubation with histaminase, while a control experiment showed that the enzyme, and not simply incubation, was the inactivating factor. Histaminase similarly inactivated an extract of the pyloric mucosa, thus confirming Ivy's results. In all cases disappearance of secretagogue powers was coincident with a loss of depressor activity. As indicated above, this latter effect was due to the presence of histamine. Since the possibility existed that the dog might have developed a conditioned reflex, an injection of saline was given, but this failed to evoke secretion.

## SUMMARY.

1. Histamine is contained in relatively large amounts in the mucous membrane of the stomach of the normal dog and in smaller quantities in the gastric muscle. Of the total amount of this substance contained in a stomach, approximately 80 p.c. is present in the fundic mucosa.

2. Extracts of dog's fundic mucosa, as well as those from pyloric tissue, have secretagogue powers which are lost after incubation of the solutions with histaminase. The results of Sacks, Ivy, Burgess and Vandolah on pyloric mucosa are thus confirmed and, in addition, it is shown that the fundic mucosa contains more secretagogue material than the pyloric. This result does not support the findings of Edkins.

This investigation was carried out in the laboratory of, and under the direction of, Prof. C. H. Best, to whom and to Dr J. G. FitzGerald, Director of the School of Hygiene, we are greatly indebted.

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THE EFFECT OF ATROPINE ON ADRENALINE  
HYPERGLYCÆMIA IN RABBITS DECEREBRATED  
ANTERIOR TO THE PONS.

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THE hyperglycæmia which results from decerebration, like that following Claude Bernard's piqûre, is supposed by some to be nothing more than a form of adrenaline hyperglycæmia due to stimulation of the adrenal glands through the splanchnic nerves. This possibility has been discussed elsewhere in the light of experiments in which it was tested by seeing whether hyperglycæmia still follows decerebration or piqûre after removal of the adrenal glands or section of the splanchnic nerves (cf. Macleod and Pearce [1912], Donhoffer and Macleod [1932], Evans, Tsai and Young [1931]). As a rule, under these conditions the blood-sugar level fails to rise, but such a result does not necessarily prove that increased secretion of adrenaline is alone responsible for the hyperglycæmia which occurs with the adrenals intact. Stimulation of glycogenolysis in the liver through the nerves supplying this organ may also be interfered with by these operations, and the possibility must not be lost sight of that a sudden cessation in the secretion of insulin may follow decerebration or piqûre. As a matter of fact, decerebration may be followed by hyperglycæmia when it is performed on an adrenalectomized animal which has been kept alive by injections of cortical extract, provided glycogen has been allowed to accumulate in the liver, as a result of rich feeding with carbohydrates.

It seemed that further evidence bearing on these questions might be obtained by a closer comparison of the types of hyperglycæmia caused by adrenaline and by decerebration, and particularly, by seeing whether they both respond in the same way to the injection of atropine. Donhoffer and Macleod showed that this drug prevented the rise in the blood-sugar percentage which otherwise occurs after pontine decerebration in rabbits in which, by previous fasting, the glycogen content of

the liver has been reduced to a low level (below 1.0 p.c.). Others have also shown that atropine suppresses or, especially when given along with ergotamine, may prevent the rise in the blood-sugar level following oral administration of sugar, but that it has no effect on the hyperglycæmia which follows the direct intravenous injection of sugar.

In view of these results it seemed worth while to compare the effects of atropine on decerebration hyperglycæmia and on that produced by injection of adrenaline and by intravenous injection of sugar. As a preliminary it was, of course, necessary to determine both the dose of adrenaline and the amount of glucose which would cause, by continuous intravenous infusion, a degree of hyperglycæmia comparable with that observed after pontine decerebration in fasted rabbits.

#### METHODS.

Rabbits which had fasted from 16 to 24 hours after being anæsthetized by intravenous injection of amytal were decerebrated in Region 2 by Schmidt's method [1923], one of the carotid arteries being left untied. At the beginning and end of the experiment a hindleg was amputated and plunged into a saline freezing mixture at a temperature below  $-10^{\circ}\text{C}$ . After an interval greater than 15 min. a representative muscle sample was cut from the frozen limb and placed directly into hot KOH for the estimation of glycogen by the Pflüger method. Liver samples for glycogen estimation were taken at the beginning and end of the experiment and placed directly in hot KOH. Blood samples were withdrawn, generally from the carotid artery but sometimes from an ear vessel, at half-hourly intervals, for estimation of glucose by the Hagedorn-Jensen method. To control any possible effect of the injection of fluid on the blood-sugar level, normal saline was injected into the jugular vein at a constant rate for some time following decerebration. When the temporary post-operative hyperglycæmia had subsided and the blood-sugar concentration had reached a relatively steady value, the saline perfusion was replaced by one of adrenaline or of glucose, the actual rate of the administration of fluid being unaltered.

Adrenaline was injected in amounts varying from 0.000104 to 0.00079 mg. per kg. per min.

Glucose was injected in aqueous solution in amounts of from 0.395 to 0.571 g. per kg. per hour in concentrations of from 8–20 p.c. In one group of experiments atropine, in aqueous solution, was administered intravenously in single doses.

## RESULTS.

The injection of saline solution alone did not have any effect on the blood-sugar level. This can be seen from the curves shown in Fig. 1, in which the results of four of the adrenaline experiments are plotted. In all of them saline solution alone was injected at a constant rate during the two hours immediately following the decerebration, which was made

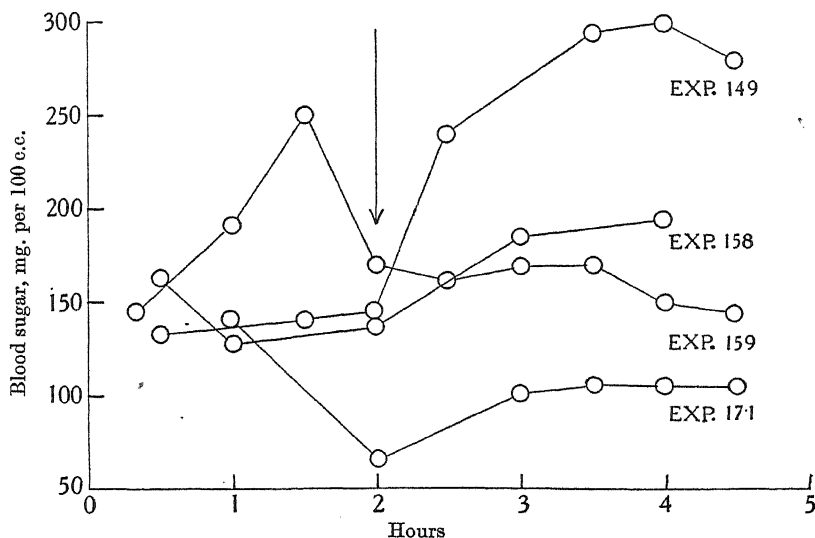


Fig. 1. The effect on the blood-sugar level of injecting adrenaline by continuous intra-venous perfusion at varying rates into rabbits decerebrated in Region 2. At arrow, saline injection was replaced by adrenaline.

Rate of injection of adrenaline:

Exp. 149.	0.00025	mg. per kg. per min.
Exp. 158.	0.000214	" "
Exp. 159.	0.00018	" "
Exp. 171.	0.000192	" "

anterior to the pons. In three experiments the blood-sugar level remained undisturbed by this operation and did not rise during the saline transfusion; in one experiment the decerebration caused an immediate rise in the level—probably because of accidental injury to the pons—but this was only temporary, the normal being regained by the third hour.

That saline injections have no hyperglycæmic effect was also noted in all the experiments in which glucose was subsequently injected. It will be convenient to consider the adrenaline experiments first.

The effect of the addition of adrenaline to the saline is illustrated in the curves of Fig. 1, which show that a degree of hyperglycæmia comparable with that which follows pontine decerebration is produced by 0.00025 mg. adrenaline hydrochloride per kg. body weight and per minute. According to the curves, it would also appear that progressively smaller doses than this have correspondingly lesser effects on the blood-sugar level. Trendelenburg and Fleischhauer [1913] found a somewhat higher rate of infusion necessary to cause glycosuria (0.0013 per min.), increase in perfusion rate being paralleled to some extent by increase in glycosuria. At about 0.0002 mg. in our experiments, no change occurred in the sugar percentage. But the grading in effect was not always manifest, being masked probably by differences in the state of carbohydrate metabolism of the animal at the time of injection. One naturally thinks of the glycogen content of liver and muscles as a dominating factor in this regard, but we have been unable, in previously fasted rabbits, to show that such is the case.

In the experiments, of which the results are given in the curves of Fig. 1, the percentages of glycogen in liver and muscles were as follows:

Glycogen in grams per 100 g. tissue.		
Exp. No.	Beginning	End
148	No estimations	
158	L. 0.902	L. 0.504
	M. 0.237	M. 0.230
159	L. 1.214	L. trace
	M. 0.861	M. 0.570
171	L. 1.318	L. trace
	M. 0.269	M. 0.259

The percentages at the start happened to be much alike for the liver, but very variable for the muscles. No conclusions can be drawn from these results regarding the behaviour of the glycogen during the periods of adrenaline injection.

Although 0.00025 mg. adrenaline usually produces marked hyperglycæmia, it may fail to do so in certain animals and we are unable to account for the difference. Thus in two experiments the following results were obtained:

	Exp. 1	Exp. 2
Average blood sugar during saline injection	0.160 p.c.	0.120 p.c.
Average blood sugar during adrenaline injection	0.090 p.c.	0.140 p.c.

The dose of adrenaline in Exp. 1 was 0.00029 mg., increased later to 0.00040 mg. In Exp. 2 the rate of injection was 0.0004 mg. per kg. per



min. It is considered permissible, however, to conclude that 0.00025 mg. adrenaline (hydrochloride) per min. and per kg. body weight will usually produce, in previously fasted rabbits, a degree of hyperglycæmia, which is comparable with that following pontine decerebration, but it must be admitted that the occurrence of exceptions detracts somewhat from the

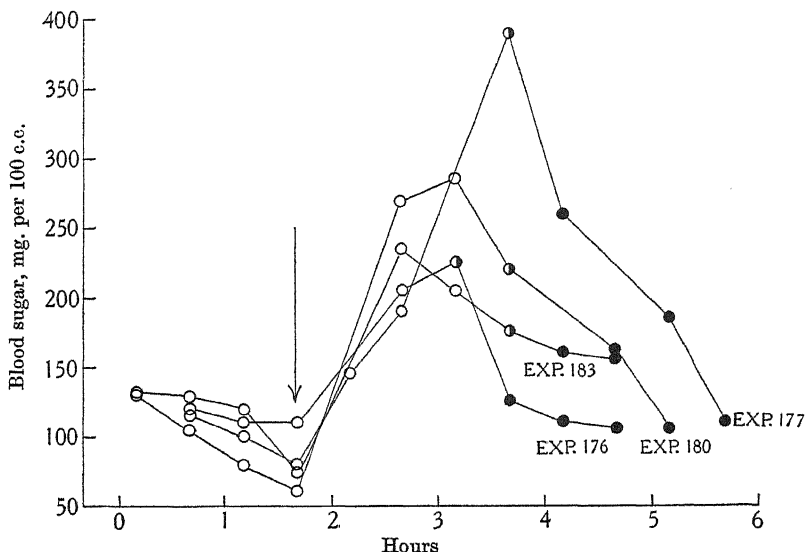


Fig. 2. The suppression of adrenaline hyperglycæmia by atropine in rabbits decerebrated in Region 2. The arrow indicates the beginning of adrenaline infusion in place of saline. Blood-sugar values after intravenous injection of a single dose of atropine are shown in black.

Rate of adrenaline injection:

Exp. 176.	0.000486	mg. per kg. per min.
Exp. 177.	0.000332	" "
Exp. 180.	0.000405	" "
Exp. 183.	0.00072	" "

value of some of our later results on the effect of atropine (*e.g.* those shown in Fig. 3).

In order to study the effect of atropine, two methods were employed: (1) injecting it after adrenaline hyperglycæmia had become established and (2) injecting it at the same time as adrenaline.

The results of four experiments of the former type are shown in Fig. 2. In two of them atropine caused the blood-sugar percentage to fall rapidly. The glycogen percentages in the liver and muscles did not

differ in their behaviour from those in the experiments in which adrenaline alone was injected, the percentage of liver glycogen being over 1.5 to start with in all cases.

The results of two experiments in which atropine was injected in large dosage at the commencement of the adrenaline injections are shown along with one in which no atropine was given in the curves of Fig. 3. Although

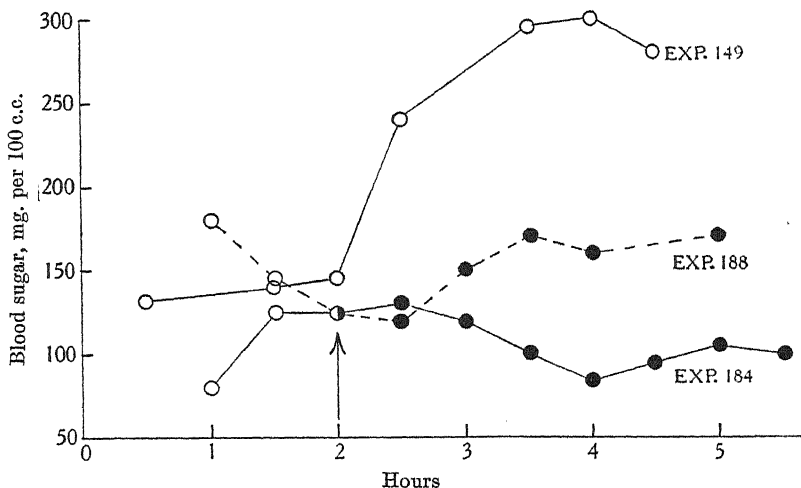


Fig. 3. The inhibition of adrenaline hyperglycæmia by atropine in rabbits decerebrated in Region 2. The arrow indicates the substitution in all three experiments of the beginning of adrenaline infusion in place of saline. It also indicates the intravenous injection of a single dose of atropine (5 mg. per kg.) in two experiments. The post-atropine blood-sugar values are shown in black.

Rate of injection of adrenaline:

Exp. 149.	0.00025 mg. per kg. per min.
Exp. 188.	0.00044      "      "
Exp. 184.	0.00079      "      "

a moderate increase in the blood-sugar percentage occurred in one of the experiments, the general effect of atropine was to inhibit the hyperglycæmic action of adrenaline, but these results are not so convincing as those shown in Fig. 2, for the reason already given. Glycogen was determined in the liver and muscles of Exp. 188, with the following results:

Liver: start 1.34 p.c.; finish, trace.  
Muscles: start 0.24 p.c.; finish 0.30 p.c.

The effect of the continuous injection of glucose solutions is shown in abbreviated form in Table I. By comparing the averages of the blood-

sugar percentages before and during injection of glucose, it can be seen that 0.4 g. per kg. body weight and per hour had no effect, whereas a decided rise occurred when 0.45 g. or more was injected. Between these two amounts it is possible that a grading in effect could be demonstrated, but our results within this range are not sufficiently numerous to make the comparison. The table (Table I) also gives the percentages of glycogen in the liver and muscles. It is significant that there is no relationship

TABLE I. Effect of continuous intravenous injections of glucose at varying rates.

Mean blood sugar during saline	Wt. of animal kg.	Blood sugar during glucose			Glycogen percentages		Rate of injection g. per kg. per hr.
		Lowest	Highest	Mean	Before	After	
155	2.42	135	250	184	L. 0.040 M. 0.441	L. Nil M. 0.224	0.413
172	2.08	280	310	294	L. 0.548 M. —	L. 0.080 M. —	0.571
180	1.35	240	310	275	L. 0.587 M. 0.500	L. 0.346 M. 0.273	0.501
213	1.70	320	400	366	L. 0.807 M. 0.397	L. 2.779 M. 0.270	0.460
142	1.95	210	300	255	L. 0.045 M. 0.497	L. 2.328 M. 0.297	0.517
160	1.5	115	220	169	L. 2.091 M. 0.398	L. 1.747 M. 0.318	0.395
185	1.27	115	220	165	L. 1.726 M. 0.357	L. 0.138 M. 0.053	0.399
246	1.37	270	330	305	L. 4.518 M. 0.439	L. 1.123 M. 0.295	0.448

between the behaviour of the liver glycogen and the amount of glucose injected. Thus, the percentage of glycogen diminished in four of the experiments in which sufficient glucose was injected to raise the average percentage of blood sugar while it increased in two of them. It diminished in the two experiments in which there was no rise in the blood-sugar level. The muscle glycogen became reduced in percentage in all of the experiments and no relationship is apparent between the extent of this reduction and the rate of sugar injection.

To investigate the effect of atropine on this form of hyperglycæmia, large doses of the alkaloid were injected after the sugar had been transfused for some time. It can be seen from the curves of Fig. 4 that this had no effect on the blood-sugar level. Glycogen estimations were made in liver and muscle as usual. In all three cases, the liver glycogen rose

from 1 to 3 p.c. The muscle glycogen did not bear any relationship to that of the liver, sometimes rising and sometimes falling.

When atropine was injected during the whole of the glucose perfusion, the results were inconstant and no conclusions are drawn from them.

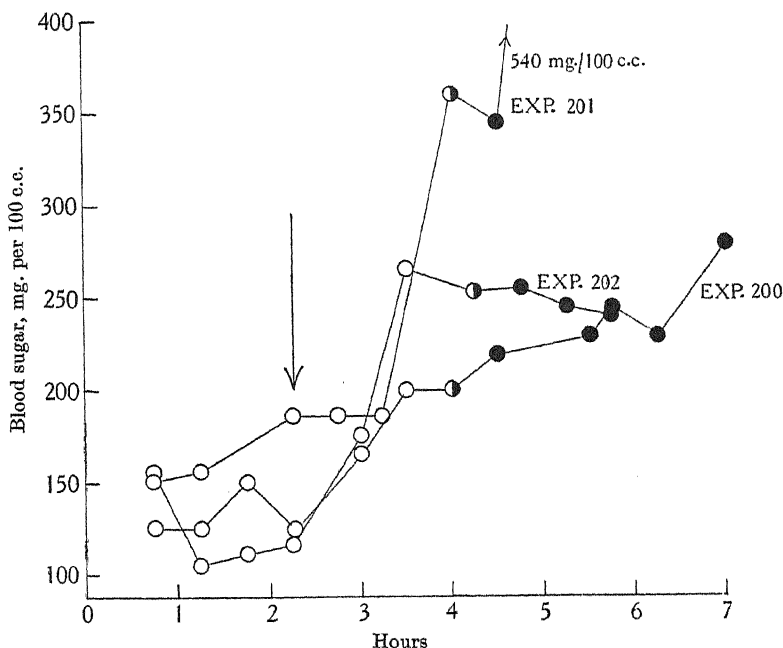


Fig. 4. The effect of atropine on the hyperglycaemia produced by the continuous intravenous perfusion of glucose into rabbits decerebrated in Region 2. The arrow indicates the beginning of infusion of glucose solution in place of saline. The post-atropine blood-sugar values are shown in black.

Rate of injection of glucose:

Exp. 200. 0.363 gm. per kg. per hour.

Exp. 201. 0.622 " "

Exp. 202. 0.664 " "

Atropine injection: 5 mg. per kg. in single dose.

#### SUMMARY.

It is undoubtedly of some significance that atropine should affect the hyperglycaemia which results from injections of adrenaline much in the same way as it does that which follows pontine decerebration or the oral

administration of sugar. If these effects were due to a retardation in the rate of glycogenolysis in the liver or to an increase in glycogen formation in this organ, it might be expected that this would be detected by a less marked decrease in the glycogen content of the organ during the course of the atropine experiments than occurred in those in which no atropine was given. But the behaviour of the glycogen content is too irregular even in the experiments without atropine to justify any conclusions being drawn from its behaviour when this was given. It would also be reasonable to suppose that if atropine promoted the formation of liver glycogen in these experiments, it would also do so when given at the same time as glucose was being added to the blood by intravenous injection, but, here again, the glycogen results are too inconstant to be of value. Even when glucose alone was added to the blood, the liver glycogens, in a long series of experiments, did not behave alike. Such results confirm the impression which is gaining ground that the conditions which determine the behaviour of glycogen in the liver are much more complex than many have supposed them to be.

It is interesting to note that the amount of glucose necessary to cause hyperglycæmia by intravenous injection in fasted rabbits decerebrated anterior to the pons is between 0.4 and 0.45 g. per kg. per hour. This is considerably less than that given by Cori [1931] for rabbits under amytal anæsthesia, viz. 2 g. The dose of adrenaline necessary to cause in rabbits decerebrated anterior to the pons a degree of hyperglycæmia comparable with that which follows pontine decerebration was usually found to be 0.00025 mg. per kg. per hour, but in a few of the experiments of this group, larger doses were required. Cori found that 0.00005 mg. per kg. per hour caused a rise in the blood-sugar level in unanæsthetized rabbits.

#### CONCLUSIONS.

1. In rabbits deprived of food overnight and then decerebrated anterior to the pons, the amount of adrenaline hydrochloride necessary to cause by continuous intravenous injection a degree of hyperglycæmia comparable with that which follows pontine decerebration, is usually 0.00025 mg. per kg. per hour. Sometimes, however, larger doses fail to have this effect.

2. Atropine injected after adrenaline hyperglycæmia has become established may cause the blood-sugar level to fall rapidly. When the alkaloid is given in one large dose at the start of the adrenaline injections, it appears to retard the rise in the blood-sugar level.

3. When glucose is added to the blood by continuous intravenous injection, it requires 0.4–0.45 g. per kg. per hour to cause a definite rise in the blood-sugar level, and atropine has no influence on this form of hyperglycæmia.

The behaviour of the glycogen of the liver and muscles in the present experiments was too inconstant to permit of any conclusions being drawn.

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## THE ALLEGED OCCURRENCE OF ACETYLCHOLINE IN OX BLOOD.

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Hampstead, N.W. 3.)*

IN spite of the closest and friendliest cooperation between Prof. Kapfhammer and ourselves, we are still unable to reach agreement on the question of the alleged occurrence of physiologically important amounts of acetylcholine in normal blood.

In a long series of experiments we have consistently failed to detect physiologically more than the merest traces of activity resembling that of acetylcholine (equivalent to not more than 0.1 mg. of acetylcholine chloride per litre of blood) in blood extracts at any stage in the process of purification, while he and his collaborators describe the detection and chemical identification of amounts up to as much as 40 mg. per litre.

It appears to be desirable to report the results of our final attempt to clear up the discrepancy, since, having regard to the present state of the literature on this question, our silence might reasonably suggest that we no longer maintained our original position.

About a year ago, a full and friendly exchange of information and experiences having failed to disclose the reason for our contradictory findings, at the invitation of Prof. Kapfhammer I went to Freiburg in the hope of discovering, by collaboration with him in his own laboratory, the explanation of our discordant results.

I will state forthwith that I had no difficulty in isolating acetylcholine from the two samples of blood examined during my visit to Freiburg. Ox blood was collected on two occasions from the municipal slaughterhouse. Dr C. Bischoff and I each worked up 1 litre of each specimen according to the Freiburg technique. This consists in precipitating the blood proteins with alcohol, and treating the extract, after concentration, with trichloroacetic acid to remove residual protein. Fat and excess of trichloroacetic acid are then removed by extraction with ether.

After appropriate concentration of the extract acetylcholine is precipitated with "Reinecke" acid, the "reineckate" is decomposed and the base converted into chloroaurate for identification.

At each stage of the process Dr Bischoff's and my results were identical in both experiments.

The two experiments were particularly remarkable in that the physiological behaviour of the acetylcholine associated with the first differed strangely from that of the acetylcholine associated with the second.

The first sample of blood was taken to the stage of purification with trichloroacetic acid before it was tested for physiological activity. The result of this test was quite concordant with our own experiments at Hampstead, for it displayed only a minute activity, equivalent to 0.1-0.5 mg. of acetylcholine chloride per litre of blood. Nevertheless, we proceeded to the next stage, namely precipitation with Reinecke acid, and most surprisingly the solution of chlorides derived from the precipitated "reineckate" possessed physiological activity equivalent to 40 mg. of acetylcholine chloride per litre of blood. There was no doubt that this activity was in fact due to acetylcholine, since the chloroaurate of the base was isolated and identified chemically.

The second sample of blood was precipitated with alcohol, and after 1 hour the alcoholic filtrate was tested physiologically, at Dr Bischoff's suggestion, before concentration on both the cat's blood-pressure and the rabbit's isolated intestine. Although the alcohol interfered to some extent in the second method, an unmistakable acetylcholine effect was observed in both, indicating approximately 22 mg. of acetylcholine chloride per litre of blood. Assayed subsequently at the trichloroacetic acid and "reineckate" stages, this extract gave equivalents of 27 and 25 mg. respectively, and acetylcholine was finally isolated and identified as the chloroaurate.

Thus acetylcholine was actually isolated from the two blood extracts and identified chemically. The appearance of the active base at the end of the fractionation of the first extract, which up to that point had given no indication that it contained more than a trace, was uncanny; particularly as the second extract displayed at every stage of purification the typical physiological activity in a degree corresponding to the amount of acetylcholine eventually isolated.

It is true that Bischoff, Grab and Kapfhammer [1931] have reported that alcoholic extracts of blood are sometimes obtained which give no physiological indication of the acetylcholine which they actually



contain until they have been treated with trichloroacetic acid, the presumption being that acetylcholine may lie hidden in such extracts as a physiologically inactive complex which is broken down by trichloroacetic acid treatment. In my first Freiburg experiment, however, this hypothetical complex apparently survived that stage, and only after precipitation with Reinecke acid did normal, physiologically active acetylcholine appear. Since a fundamental disagreement still exists between Kapfhammer's results and our own, it is superfluous to discuss this hypothetical complex. Apart from the chemical difficulties which it presents, its inconstancy in appearance and behaviour is perplexing; and in our investigations at Hampstead we have never encountered it.

Returning from Freiburg to Hampstead, I again examined ox blood from four different animals, using the technique with which I had become familiar in Kapfhammer's laboratory. In none of the extracts was more than a trace of acetylcholine-like activity (equivalent to not more than 0.1 mg. of acetylcholine chloride per litre of blood) physiologically detectable at any stage in the fractionation, which in each experiment was carried to completion in spite of the apparent absence of acetylcholine. The gold salts finally obtained consisted mainly of choline chloroaurate.

In order to test my technique, the unconcentrated alcoholic extract of the fourth sample (2 litres) of blood was divided into two equal quantities, to one of which I added 40 mg. of acetylcholine chloride. The two portions were then worked up simultaneously. Without difficulty, and with relatively little loss, the added acetylcholine was recovered, and finally identified as chloroaurate.

For the complete failure of the experiments made in this laboratory to confirm the positive results of those performed under precisely the same technique in Freiburg, we can find no reasonable explanation.

It should be mentioned that, since this work was completed, a short note by Vogelfanger [1933] has appeared, stating that there is no difficulty in obtaining from ox blood, by Kapfhammer's method, acetylcholine in amounts similar to those which he obtained. The author notes that the amount of oxalic acid recommended by Kapfhammer is inadequate to ensure a proper acidity of the initial extract, and emphasizes the necessity of adjusting the amount to that end. It might be implied that the failure of others to find acetylcholine in ox blood was attributable to neglect of this point. This criticism does not apply to our earlier work, for we ourselves discovered this defect in Kapfhammer's original directions, when applied to English ox blood, and took the

necessary steps to correct it [Dale and Dudley, 1931]. Apart from this critical note on a point already recognized and dealt with by us, Vogelfanger's account is a bare record, and does not assist to an understanding of our negative findings. Our numerous and consistently negative results prove that acetylcholine cannot be an artefact, and the erratic appearance of the substance in physiologically detectable form at different stages of the fractionation in different experiments rules out the improbable suspicion that there might be occasional batches of alcohol current in Germany which were contaminated with acetylcholine.

Since the same technique gives me positive results, confirming Kapfhammer's, in Freiburg, and negative results, confirming all our earlier observations, in London, it is obviously desirable that the question should be tested by others as widely as possible. Already Wrede and Keil [1931] have described negative results and Vogelfanger positive.

I earnestly hope that the publication of this paper will stimulate others, in this country and abroad, to investigate the problem. The method of Kapfhammer and Bischoff is admirably suited to its purpose, and has transformed the process of isolation of acetylcholine from one of great labour and difficulty to one of ease and simplicity.

In the following paper Chang and Gaddum publish experiments made on extracts of other tissues by physiological methods. Only in one case, that of the human placenta, as earlier in the case of horse and ox spleen, do these further experiments detect quantities of acetylcholine comparable to those found by the Freiburg investigators. With all other tissues, the experience of this laboratory entirely fails to confirm the presence of still higher proportions (nearly 200 mg. per kg. in some cases) of acetylcholine found by Kapfhammer and his collaborators.

#### EXPERIMENTAL.

The method of Kapfhammer and Bischoff, employed in the experiments under discussion, was carried out in the following manner in Freiburg.

Ox blood was collected at the municipal slaughter-house, where oxalic acid (1.6 g. per litre of blood) in saturated aqueous solution was added. It was then brought to the laboratory, a journey of about 20 min. duration, where 1 litre was poured with stirring into 5 litres of 96 p.c. alcohol. The reaction of the mixture was tested with blue litmus paper. As no definite acid reaction was detectable, aqueous lactic acid was added until the solution produced a faint reddening of litmus.

After standing for  $1\frac{1}{2}$ –2 hours the solution was filtered through paper and mixed with the liquid expressed in a tincture press from the blood coagulum. The blood cake was re-extracted with 2 litres of 96 p.c. alcohol, which, after 2 hours' contact, was filtered off. The combined extract was then evaporated *in vacuo* (water bath at 40°) to 100–150 c.c.

An equal volume of 20 p.c. trichloroacetic acid was added to this concentrate and the mixture was placed in an ice-chest (4°) for 2 hours. It was then shaken out with ether four times, and the solution, now faintly acid to Congo-red, was concentrated *in vacuo* to approximately 100 c.c. Saturated aqueous Reinecke acid was added in slight excess and the mixture was placed in the ice-chest for 12-16 hours.

The precipitate was then filtered off and washed with ice-cold water until the runnings were practically colourless. Ice-cold alcohol was then applied; the runnings were at first faintly coloured, but speedily became colourless. After a final washing with ether the residual precipitate was dried in a desiccator. It was then dissolved in 25-50 c.c. of dry acetone. The solution, after removal of a small amount of insoluble material by filtration, was mixed with dry benzene (10-15 vols.). This precipitated the "reineckate" quantitatively, and it was collected on a folded filter paper. After drying, it was dissolved in about 100 c.c. of 50 p.c. acetone, excess of aqueous silver sulphate solution was added, the precipitate of silver "reineckate" removed in the centrifuge and to the liquid a solution of barium chloride, equivalent to the silver sulphate used, was added. The solution, freed from silver chloride and barium sulphate in the centrifuge, was concentrated *in vacuo* to 5 c.c. A strong solution of gold chloride, neutralized to Congo-red with sodium hydrogen carbonate, was added to this solution. After standing in the ice-chest for about an hour the gold salt was filtered off and recrystallized from water.

The experiments made at Hampstead were carried out strictly in accordance with the technique just described. The amount of lactic acid added to each alcoholic filtrate was 1 c.c.

The "reineckate" was precipitated from acetone solution by means of benzene for the sake of strict conformity, but this step does not appear to serve any useful purpose and could be omitted.

Table I shows the amounts of acetylcholine determined physiologically at each stage of the fractionation of the two Freiburg specimens and of the fourth sample of English ox blood, which was worked up in two equal

TABLE I. Acetylcholine in ox blood (mg. acetylcholine chloride per litre).

	Freiburg experiments		Hampstead experiments	
	Blood I	Blood II	Blood IV a	Blood IV b
Alcoholic extract before conc.	—	22 (approx.)*†	—	—
Alcoholic extract after conc.	—	—	0.13†	40†
After trichloroacetic acid purification	0.1-0.5*	27†	0.13†	40†
After Reinecke acid precipitation	40†	25†	{ 0.09† 0.05‡	{ 33.3† 34.4‡
Weight of chloroaurate after recrystallization	50 mg. (M.pt. 162°; Au. 40.4 p.c.)	30 mg. (M.pt. 163°; Au. 40.4 p.c.)	40 mg. (M.pt. 258°; Au. 44.3 p.c.)	54 mg. (M.pt. 164°; Au. 40.5 p.c.)

Physiological determination: \* Cat's blood-pressure; † Rabbit's intestine; ‡ Frog's eserized rectus. Au. calc. for acetylcholine chloroaurate, 40.6 p.c.; for choline chloroaurate, 44.5 p.c.

portions, to one of which (IV b) 40 mg. of acetylcholine chloride had been added. The other three English ox bloods gave results similar to those of IV a. When such small amounts of acetylcholine as appear in blood IV a are under examination the frog's eserinizied rectus gives a much more accurate assay than does the rabbit's intestine. With the latter a fraction of the total activity still persists after atropinization of the test tissue. The amounts of twice recrystallized chloroaurate, with melting points and analyses, are also presented. The crude chloroaurate obtained from blood extracts always contains the choline salt. When the acetylcholine salt is also present the volume of water used for recrystallization is such that the choline salt remains in the mother liquor, since acetylcholine chloroaurate is much less soluble than choline chloroaurate.

It is to be noted that the exhaustive washing of the "reineckate" results in a small loss (15 p.c.) of acetylcholine.

#### SUMMARY.

Kapfhammer and his colleagues in Freiburg maintain that ox blood contains relatively large amounts of acetylcholine, whilst we at Hampstead have consistently failed to confirm their finding.

This fundamental discrepancy persists in spite of a collaborative attempt, described in the present communication, to discover a reason for our disagreement.

In conclusion I wish to record my appreciation of the friendliness with which Prof. Kapfhammer received me in his laboratory, and of the expert and willing advice and assistance afforded me by Dr Bischoff in making my experiments there.

I am indebted to Sir Henry Dale, Dr J. H. Gaddum and Dr H. C. Chang for their help in making the necessary physiological determinations.

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## CHOLINE ESTERS IN TISSUE EXTRACTS.

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SINCE Loewi [1921-30] brought evidence to show that the vagus nerve inhibits the frog's heart by liberating a substance pharmacologically identified as a choline ester, similar evidence has been obtained in experiments with a number of other nerves. It has been shown that the same mechanism is probably responsible for the action of the third nerve on the eye [Engelhart, 1931; Velhagen, 1931, 1932] of the "anti-dromic" vasodilator nerves to voluntary muscle [Dale and Gaddum, 1930; Feldberg, 1932; Bain, 1932], of the chorda tympani on the secretory cells of the submaxillary gland [Babkin, Stavratsky and Alley, 1932; Babkin, Gibbs and Wolff, 1932; Gibbs and Szelöczey, 1932 *a, b*; Henderson and Roepke, 1932], and of the splanchnic nerve on the suprarenal medulla [quoted from Feldberg, 1933]. There is also some reason to believe that the intestine is capable of esterifying choline in the presence of the appropriate anions to form highly active choline esters [Le Heux, 1919; Magnus, 1930], and that this mechanism is under control of the vagus [Hoet, 1925]. Fuller reference to work on this subject will be found in papers by Riesser [1931], Hirschberg [1931] and Kroetz [1931].

In 1925 Witanowski showed that a substance with the pharmacological properties of a choline ester could be obtained from frog's heart by mincing the tissue in alcohol. This observation has frequently been confirmed and similar results have been obtained with other tissues.

The only choline ester which has hitherto been isolated in a chemically pure state from animal tissues is acetylcholine, which was found to be present in large quantities in the spleens of horses and oxen [Dale and Dudley, 1929, 1931]. More recently Bischoff, Kapfhammer and

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their co-workers [1930, 1931, 1932] (see also Vogelfanger [1932]) have announced the isolation of even larger quantities of acetylcholine from blood, skeletal muscle, and many other tissues. Repeated attempts have been made in this laboratory to confirm these observations regarding blood and skeletal muscle and these attempts have been unsuccessful. We have, however, developed improved methods of making tissue extracts and testing them for acetylcholine.

In the discussion of our results we have found it convenient to use two new terms. The term "A.C.-substance" means "a substance pharmacologically identified as acetylcholine." "A.C.-equivalent" means "the apparent concentration of acetylcholine in terms of the chloride."

#### THE PREPARATION OF EXTRACTS.

The conditions necessary for obtaining acetylcholine in extracts of horse's spleen have been discussed by Dale and Dudley [1929]. The spleen may be removed from the horse and kept intact for some hours without appreciable loss of acetylcholine, but as soon as the tissue is cut up or minced for extraction the acetylcholine rapidly disappears, and appreciable loss may occur even if the tissue is allowed to drop directly from the mincer into alcohol. In order to obtain maximum yields it is therefore important that the tissue should be soaked in some liquid which will inhibit the disappearance of activity, and disintegrated in this liquid.

The liquid which has most frequently been used in the preparation of such extracts is ethyl alcohol. Since it is known that acetylcholine is most stable in weak acids ( $pH$  3-9, Hofmann, 1930) some workers add 1 c.c. of normal hydrochloric acid to each 100 c.c. of alcohol. We have obtained more satisfactory results with sulphuric acid, since extracts containing this acid filter more readily than extracts containing hydrochloric acid. In an experiment with dog's liver it was found that the final solution was of about the right acidity when each 100 g. of tissue was extracted with 400 c.c. of alcohol containing 5 c.c. of  $2N$   $H_2SO_4$ . A similar extract was then prepared from a horse's spleen and compared for activity on the rabbit's intestine with an extract made without the addition of acid. In this way it was found that the yield of acetylcholine was slightly increased by the addition of this quantity of sulphuric acid.

Trichloroacetic acid was used by Galehr and Plattner [1928] to precipitate the proteins in blood and thus stop the destruction of acetylcholine. In three experiments we have found that extracts of horse's spleen and intestine prepared by means of this acid contained 12-30 p.c.

more activity than similar extracts prepared from the same tissue with acid alcohol as described above, and we have therefore adopted the following method of preparing extracts:

The tissue is weighed and placed in 10 p.c. trichloroacetic acid (2 c.c. of acid for each g. of tissue). It is cut up in the acid with scissors and then left with occasional stirring for 1-2 hours. The extract is filtered through paper on a Buchner filter and the tissue washed with 7 p.c. trichloroacetic acid. The extract is then shaken four or five times with ether in a separating funnel until it is only faintly acid to congo. The extract is usually left at this stage in the cold room overnight. It is then concentrated at low pressure at about 40° until 1 c.c. of extract corresponds to 1-10 g. of tissue. This solution is made neutral to congo-paper and then used in the biological test. It was found that cooling the acid and tissue to 5° before disintegration resulted in no further increase in the yield, and the extracts have therefore been prepared at room temperature.

It is known that the esterase which destroys acetylcholine is inhibited by eserine, and it is therefore possible that, if eserine were injected into an animal before it was killed, larger yields of acetylcholine would be obtained from its tissues. However, it is improbable that this would be so when the tissue is disintegrated beneath the surface of a solution of trichloroacetic acid. The acetylcholine in a horse's spleen appears to be stable until the tissue is disintegrated, and very little destruction can occur in the fraction of a second which elapses after a piece of tissue is cut and before the enzyme is inhibited by the trichloroacetic acid. We have not adopted previous eserination as a routine procedure because it was desirable that the extracts of tissues should be free from traces of eserine. On the one occasion on which an extract was made from the submaxillary gland of an eserinated dog, the yield of acetylcholine activity was below the average of all the results obtained with the submaxillary glands of normal dogs.

Extracts prepared in the way described above contain large quantities of other pharmacologically active substances besides acetylcholine, and are only suitable for use in a biological test when the physiological reaction used in this test is one which is relatively little affected by such substances as potassium, histamine and adenosine compounds. For experiments with the frog's heart it is necessary to purify the extracts further, and the methods which are used for this purpose may lead to the partial disappearance of acetylcholine. Thus, in recent papers, Plattner and Krannich [1932] describe experiments in which they used the frog's heart to test quantitatively for acetylcholine in extracts of frog's voluntary muscle. The tissue was extracted with acid alcohol and the extract was taken down to dryness on a water bath. The residue was then shaken with absolute alcohol and again filtered. This process was repeated

several times and the final residue was taken up in Ringer's solution and tested. It seemed to us to be unlikely that acetylcholine would survive repeated evaporation to dryness on a water bath, and we have carried out an experiment to test this point. For this purpose we tested extracts of horse's spleen in comparison with acetylcholine, by means of the rabbit's isolated intestine. The experiment was carried out in this way, because we know with certainty that the effect of this extract on this tissue is mostly due to acetylcholine. Two extracts were prepared—one by Plattner and Krannich's method, and the other by the method using trichloroacetic acid, described above. Both extracts contained a substance which stimulated the intestine and which was easily destroyed by alkali, but the yield obtained by Plattner's method was only about 1/30 of that obtained by the other method. We concluded that 97 p.c. of the acetylcholine originally present in the tissue was lost in carrying out Plattner and Krannich's method of purification. It is not, of course, possible to conclude from this that similar losses would occur when the same method of extraction is applied to extracts of voluntary muscle, and we therefore do not wish to suggest that the active principle studied by Plattner and Krannich was not acetylcholine. Indeed, they present evidence that no other substance hitherto isolated from animal tissues could be responsible for the effects which they observed. Nevertheless, we do not think that this method of extraction can give a reliable estimate of the original acetylcholine content of tissues.

#### THE BIOLOGICAL TESTING OF EXTRACTS.

A large number of different pharmacological preparations are affected by small doses of acetylcholine, and many of these are suitable for testing extracts of horse's spleen, which contains so much acetylcholine that the presence of other substances does not affect the result. However, the extracts which we have prepared from other tissues did not contain these large quantities of acetylcholine, and some of the methods which we have tried have proved unsuitable because the effect of tissue extracts on them was largely due to substances other than acetylcholine. The difficulties encountered in using some of these preparations are discussed below.

##### *The frog's heart.*

Straub's preparation of the frog's heart has been used by various investigators in testing extracts for acetylcholine. If a constant volume (say 0.1 c.c.) of different dilutions of the extract is added to the solution



(ca. 1 c.c.) in the cannula until a concentration is found which produces a definite decrease in the size of the beat, extracts can be compared quantitatively with reasonable accuracy (10–20 p.c.) by giving the two solutions in alternate doses. Different hearts vary rather widely in their sensitivity, but it is usually possible by this method to detect doses as small as 0.01  $\gamma$  of acetylcholine, or less. The frog's heart is also affected by a very large number of other substances which are known to be present in tissue extracts. Small quantities of acid produce an effect similar to that of acetylcholine, so that extracts must be carefully neutralized before they are tested. This introduces a further complication, since acetylcholine is unstable unless the solution is kept slightly acid. Potassium (0.25 mg. KCl) produces an effect very similar to that of acetylcholine. An increase in the size of the beat may be produced by calcium or histamine, or by some substance associated with various complex fats [Clark, 1913; Eggleton, 1926], and the presence of these substances might mask the presence of acetylcholine. Adenosine, adenylic acid and adenylypyrophosphate appear to have no effect on the size of the beat, but they slow the heart and may stop it altogether if they are not removed from extracts.

Effects due to choline and its esters can be distinguished from effects due to any other substance known to occur in the body by the fact that they are antagonized by atropine. We have not found this test to be always easy to apply, because the sensitivity of the preparation to acetylcholine often decreases during the course of an experiment, even in the absence of atropine, when the preparation is treated with various tissue extracts. In any case it is impossible to carry out an assay of an extract for choline esters in the presence of significant quantities of the other substances mentioned, and this fact has led to the use of rather drastic chemical procedures for the removal of these undesirable substances. We have already discussed the probability that some of the procedures which have been used may also remove a large proportion of the acetylcholine when it is present.

Unknown substances may also interfere with the use of the frog's heart in testing for acetylcholine. Evidence for this statement is shown in Fig. 1 which shows the effect of an extract of rabbit's voluntary muscle which had been subjected to several stages of purification. The original extract had been made with trichloroacetic acid. This had been shaken with ether, and then taken down to a small volume. Nine volumes of alcohol were then added and the precipitate filtered off. The alcohol was removed from the filtrate, which was then treated with Reinecke

acid to remove choline and other bases. The filtrate was treated with silver sulphate to precipitate the excess of Reinecke acid, and then with  $\text{BaCl}_2$  to precipitate silver sulphate. Lastly an excess of sodium sulphate had been added to remove barium, and the solution was neutralized to litmus. The tracing (Fig. 1) shows that it contained a substance which inhibited the frog's heart in much the same way as acetylcholine though its action persisted when the heart was rendered insensitive to acetylcholine by means of atropine. The solution was also tested by means of the frog's rectus as described below, and it was found to contain no detectable acetylcholine. A blank control (B.C.) was carried out which

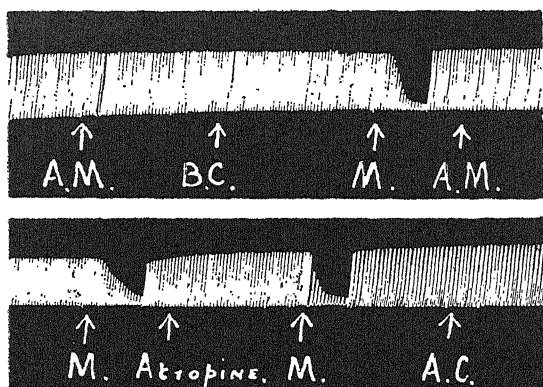


Fig. 1. Frog's heart (Straub's method). *M.* Purified extract of voluntary muscle (0.25 g. tissue). *A.M.* The same after ashing. *B.C.* Blank control (reagents alone). *A.C.* Acetylcholine (0.05γ).

showed that the active substance was not derived from the reagents. We have at present practically no evidence as to the nature of the substance responsible for this effect, which we refer to as substance *R*. It is shown in Fig. 1 that substance *R* is destroyed by ashing and is therefore not potassium. When an extract was boiled for 5 min. with an equal quantity of 2*N* NaOH substance *R* was partially, but not completely, destroyed. When the extract was similarly treated with HCl a smaller proportion of the activity was destroyed. When an extract was taken to dryness and shaken with absolute alcohol substance *R* was detected in the alcohol.

The properties of substance *R* are such that we are unable at present to devise any simple method of removing it from extracts and leaving the acetylcholine intact. The procedures used by Plattner and Krannich

[1932], and discussed above, would be likely to preserve substance *R*, and we do not know why this substance did not inhibit the frog's heart, in their experiments, after atropinization. If this substance is present in frog's muscles in as high concentration as in rabbit's muscle it would certainly be expected to do so. Plattner and Krannich found that the effect of their extracts was abolished by atropine, that the active substance was destroyed by blood, and that acetylation did not increase the activity to such a large extent as that of choline is increased. Acetylcholine is the only substance which is known to occur in animal tissues, and to have this combination of properties, and there is no reason to doubt that the effects observed by Plattner and Krannich were, in fact, due to acetylcholine. We have not investigated this point more closely, because we believe that the evidence already obtained clearly indicates that the methods used by Plattner and Krannich give a less reliable indication of the acetylcholine content of animal tissues than other methods described later in this paper.

*Rabbit's intestine and rabbit's blood-pressure.*

These two preparations, unlike the corresponding preparations prepared from cats, are very insensitive to histamine, and we have therefore tried to use them as test objects for acetylcholine. They have been found unsuitable owing to the difficulty of excluding the complicating effects of two groups of substances:

(1) Adenosine and its derivatives which inhibit the intestine and depress the blood pressure. These substances can, however, be almost completely removed by taking advantage of the fact that they are insoluble in high concentrations of alcohol.

(2) An unknown substance which we call substance *P*—some of whose properties have already been described [Euler and Gaddum, 1931]. This substance depresses the blood-pressure and stimulates the intestine so that in these respects it simulates acetylcholine, but its effects are not abolished by atropine.

*Cat's denervated gastrocnemius.*

About a week after preliminary aseptic section of the sciatic nerve the cat's gastrocnemius becomes sensitive to acetylcholine and other drugs allied to nicotine [Frank, Nothmann and Hirsch-Kauffmann, 1922; Dale and Gasser, 1926]. This phenomenon may be used as the basis of a sensitive and specific test for acetylcholine in tissue extracts.

The following are the details of the technique which we have found to be most satisfactory.

The preliminary, aseptic nerve section is performed under ether anaesthesia a week before the experiment. The cat is then anaesthetized with chloralose and artificial respiration is applied. The last lumbar arteries and all lower branches of the aorta except the two external iliac arteries are tied. The denervated gastrocnemius is freed, and the femoral artery is tied immediately below it. The tension is recorded isometrically and the optimum initial tension is determined at the beginning of each experiment. Warm Ringer's solution is made to flow continuously over the muscle. Eserine (0.2 mg.) is injected intravenously to increase the sensitivity, and atropine (5 mg.) is injected subcutaneously to abolish the vasomotor effects of acetylcholine, which are liable to cause variations in the response. Atropine does not affect the reaction under study. Injections are made into the base of the aorta through the central end of the external iliac artery running to the normal leg by means of the special device illustrated in Fig. 2. This consists of a small cannula into which is inserted a rubber cap, slit at top so as to act as a valve. In the figure a brass fitting, made from one of the needles supplied with the syringe, is shown inside the rubber cap. This was found to be a convenient addition to the apparatus, but in most of the experiments injections were made by fitting the nozzle of the syringe directly into the rubber cap. The dead space between the valve and the artery is only about 0.05 c.c., and this is negligible if the volume of the injection is 0.5 c.c. or more. Small quantities of active substances which might remain in this space are washed out by injecting saline in between each main injection. Blood is rather liable to enter the mouth of the cannula between injections and clot there. This tendency can be largely avoided by using cannulae whose mouths are reduced to a hole so small as to be scarcely visible, but consistently satisfactory results have only been obtained when the cat has received a preliminary injection of heparine. The main advantages of this type of cannula are that none of the injected solution is wasted except the quantity required to fill the inside of the rubber cap (about 0.1 c.c.) and that, if the cannula is fixed in a clamp, injections can be made without mechanically disturbing the cat. Injections can be made at intervals of about 5 min. In satisfactory preparations doses of 0.1 $\gamma$  or 0.2 $\gamma$  of acetylcholine cause consistent reactions under these conditions.

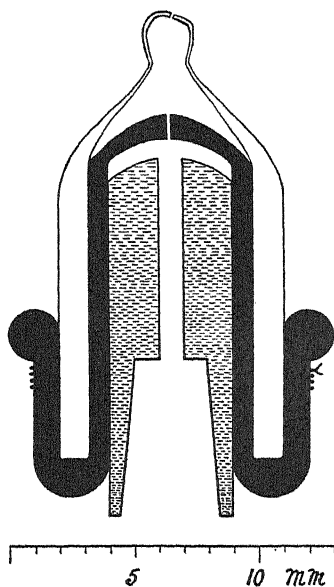


Fig. 2. Glass cannula for intra-arterial injections, containing rubber cap to act as valve, and brass fittings to receive nozzle of syringe.

The result of a comparison carried out in this way is shown in Fig. 3. The continuous straight line in this figure shows the effect that would presumably have been produced by 0.24 $\gamma$  of acetylcholine. It will be seen that 0.6 c.c. of the test solution had less than this effect and 0.9 c.c.

had more. The preparation is fairly sensitive and appears to be at least as specific as any other known test for acetylcholine. It is presumably unaffected by the presence of atropine in the extract tested. Its chief disadvantage lies in the fact that it is excessively laborious.

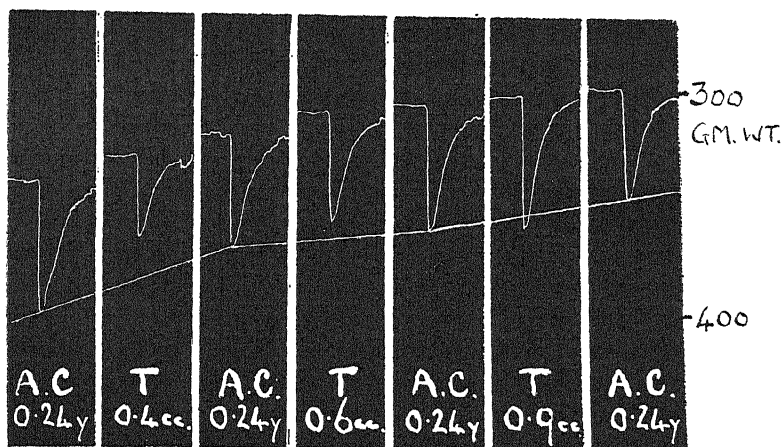


Fig. 3. Cat. Atropine and Eserine. Tension of denervated gastrocnemius. Unknown solution, T, compared with a constant dose of acetylcholine.

#### *Frog's rectus abdominis and leech muscle.*

The frog's rectus abdominis and the longitudinal muscle of the leech (*Hirudo medicinalis*) both provide sensitive and specific test objects for acetylcholine in tissue extracts. In both cases the preparation is specifically sensitized to acetylcholine by treatment with eserine, so that this drug may be used to distinguish acetylcholine from other substances which might be present in extracts.

The action of acetylcholine on the frog's rectus was first described by Riesser [1921] and has since been widely studied, but has, apparently, not been used in the testing of tissue extracts. The muscle obtained from *Rana esculenta* is rather more sensitive than that obtained from *R. temporaria*, but either species can be used.

The action of acetylcholine on the eserinizied leech was first described by Fühner [1918*a, b*]. The use of this preparation for the assay of extracts for acetylcholine was recommended by Minz [1932] while the present work was in progress. The reaction is unaffected by atropine and abolished by nicotine. In our experience the leech has not proved such a satis-

factory test object as the rectus, but it may be used with advantage to confirm the results obtained with the rectus, and as the two preparations have much in common it is convenient to describe them together.

The bath we have used for both preparations contains 18 c.c. of Ringer's solution at room temperature. The magnification was about 10 and the tension about 3 g. wt. for the rectus, and more for the leech. The doses used were only slightly greater than the threshold dose. In both cases the action of acetylcholine is a very slow one, and we have found that two solutions of acetylcholine can be satisfactorily compared in a relatively short time by means of the following device. The drug dissolved in a volume of less than 0.5 c.c. is added to the bath and left in contact with the preparation for exactly 3 min. (or, with some preparations, exactly 2 min.). After this time the effect is usually not quite complete, but, nevertheless, the solution is changed and the preparation allowed to relax. If doses are added at a constant time interval of 10 min. the effect produced in the given constant time is regularly related to the dose, and can be taken as an index of the potency of the solution. When a small effect has been obtained with the extract on the normal preparation, 0.2 mg. of eserine sulphate is added to the bath so as to give a concentration of eserine of about  $10^{-5}$ . This concentration is more than sufficient to give a nearly maximal sensitization to acetylcholine, but this sensitization takes about half an hour to develop. If the extract contains detectable quantities of acetylcholine its action is then very definitely increased, and a quantitative estimate of the acetylcholine equivalent of the extract is obtained by comparing it with a solution of pure acetylcholine given in alternate doses. 0.2 mg. of eserine sulphate is always added to the bath after the solution has been changed. This is more satisfactory than adding the eserine to the main bulk of Ringer solution, because eserine is not very stable in high dilutions, except in the presence of acid.

Fig. 4 shows the action of a series of substances on the frog's rectus. 12 mg. of KCl, 2γ of acetylcholine and 2 mg. of choline produced, in this experiment, effects of roughly equal size, but of very different shapes. These shapes are characteristic of these three substances. Potassium causes an initial twitch followed by a slow contracture, and acetylcholine differs from choline in that its effect starts more quickly and describes a curve which is more convex upwards. The last three effects were obtained after eserine had been added to the bath. The actions of choline and potassium were practically unchanged, while that of acetylcholine was increased.

The leech preparation differs from the rectus preparation in the following aspects:

(1) In our experience it was usually difficult to obtain a satisfactory

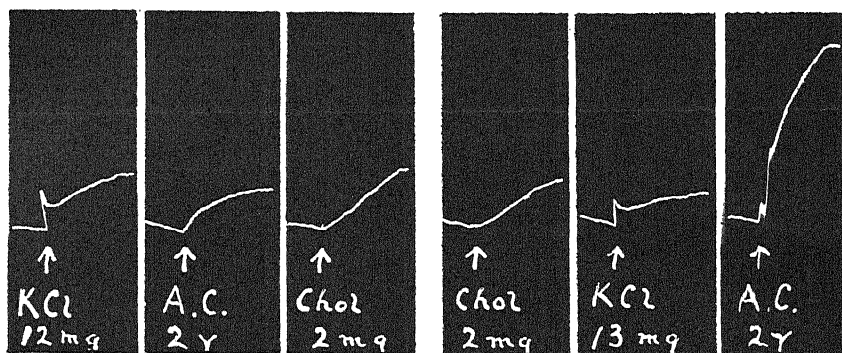


Fig. 4. Frog's rectus abdominis in 18 c.c. bath. First three doses—no eserine. Last three doses  $10^{-5}$  eserine sulphate.

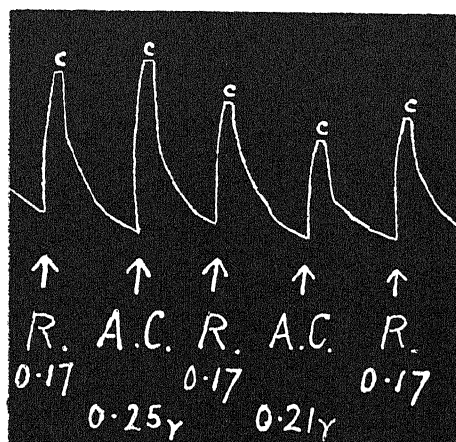


Fig. 5. Frog's rectus abdominis. Eserine sulphate  $10^{-5}$ . R. Extract of dog's right submaxillary gland. Dose in g. of tissue. A.C. Acetylcholine.

comparison with the leech because of wide variations in the length to which the preparation relaxed when the drug was washed out.

(2) Before eserine, acetylcholine has no effect on the leech, except in very large doses. After eserine the leech is slightly more sensitive than

the rectus. A specially reactive rectus is then about as sensitive as an unusually insensitive leech. The effect of eserine on the leech is thus very much greater than its effect on the rectus.

(3) Potassium causes a contraction of the rectus, but a relaxation of the leech. The smallest dose which produces these effects is about the same in the two cases. There is thus no danger of confusing the effect of

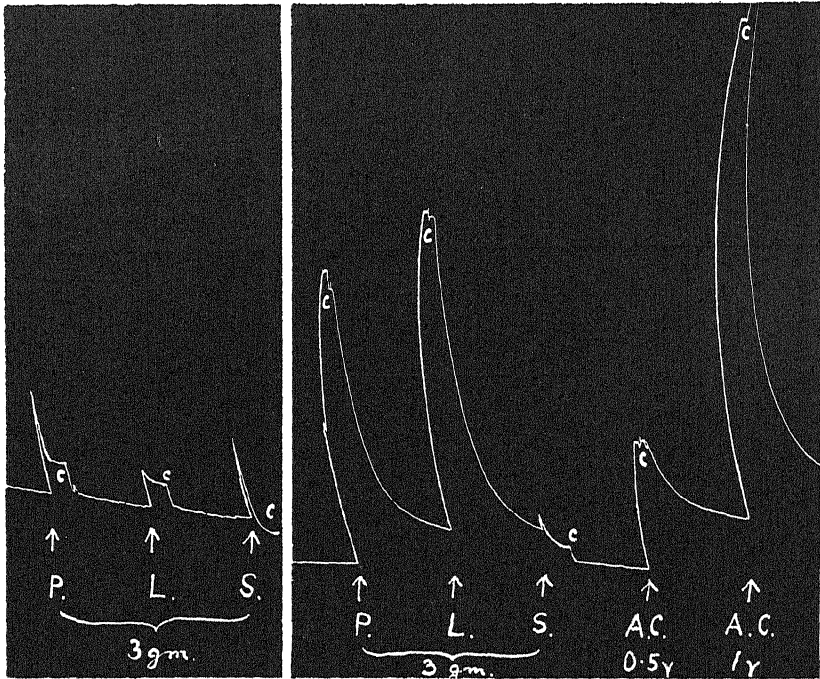


Fig. 6. Frog's rectus abdominis. Eserine sulphate ( $10^{-5}$ ) added between the two sections of tracing. Extracts of dog's pancreas, lung and spleen. Dose in g. of tissue. A.C. Acetylcholine.

potassium on the leech with that of acetylcholine, but a large amount of potassium may mask the presence of a small amount of acetylcholine. In some cases where the suspicion has arisen that effects observed on the rectus were due to potassium, the suspicion has been confirmed by testing the extract on the leech.

Histamine, adenosine, calcium, and the substances *P* and *R* discussed above, given in large doses, had no action on either preparation.



Fig. 5 shows a comparison carried out with an eserinizied rectus. *R* represents an extract of a dog's right submaxillary gland. The plateaux at the maxima of the effects represent the times during which the lever was arrested and the solution changed. In this experiment the volume of extract corresponding to 0.17 g. of gland was equivalent to something between 0.25 $\gamma$  and 0.21 $\gamma$  of acetylcholine.

Fig. 6 shows the result of some tests near the limits of the sensitivity of the method. The first three doses correspond to 3 g. of a dog's pancreas, lung and spleen. The small effects obtained were probably due to potassium. The preparations were now eserinizied and it was found that the pancreas and lung contained some detectable acetylcholine-like substance, but the spleen did not. It was subsequently found that if the spleen had contained as much as 0.1 $\gamma$  of acetylcholine per g. this quantity would have been detected in this test.

#### THE PHARMACOLOGICAL DIFFERENTIATION OF ACETYLCHOLINE FROM OTHER SUBSTANCES.

The following special tests have been used to differentiate between effects due to acetylcholine and those due to other substances.

(1) If the action of an extract is increased by eserine this fact is in itself sufficient to justify the conclusion that the effect is not due to choline—or to any other substance, except acetylcholine, which is known to be present in tissues. This is certainly true for tests on the frog's rectus and the leech, but eserine cannot always be used in this way when other pharmacological preparations are used. For example, it has been found impossible to apply sufficient eserine to potentiate a rabbit's isolated intestine without producing a contraction due to the eserine itself, or, more probably, to potentiation of the action of a choline ester naturally present [Matthes, 1930].

(2) The activity should disappear rapidly when the extract is mixed with blood, but if the blood has been previously treated with eserine this reaction should be very greatly slowed. In applying this test it is sometimes found that the rate of destruction is different from the rate of destruction of pure acetylcholine under the same conditions. This observation does not in itself prove that the original effect was not due to acetylcholine, because it is possible that the rate of destruction is affected by other substances, such as choline, which may be present in the extract.

(3) The active principle should be unstable in alkaline solution. If a portion of an extract is mixed with an equal volume of 2N NaOH, kept

for 10 min. at room temperature, and then neutralized, any acetylcholine which it contains is destroyed, while choline is unaffected.

(4) The choline content of the extract may be determined by acetylating it and then comparing it pharmacologically with acetylcholine. It is possible to calculate, from the result of this test, whether the amount of choline originally present was sufficient to account for the action originally observed. Calculations of this type give no information about the active substance under study, except that it is not choline—a fact which can usually be proved by other simpler tests.

(5) Many of the effects of acetylcholine are antagonized by atropine. Other effects are antagonized by nicotine. The extract should react to these drugs in the same way as acetylcholine itself. Tests of this kind have served to differentiate effects due to histamine, adenosine compounds, potassium, and the substances *P* and *R* discussed above.

(6) When the activity of the extract is estimated quantitatively in terms of acetylcholine, using several different pharmacological tests, the same result should be obtained in each case.

The last of these methods probably provides much stronger evidence for the pharmacological identification than any of the others. For example, it serves to differentiate acetylcholine from other choline esters. The fact that the different kinds of activity which these substances possess may vary independently is well known (see, for example, Simonart, 1932), but in order further to illustrate the point we have compared several different esters with acetylcholine by five different methods. The results of these experiments are shown in Table I in which the apparent activity of each ester is expressed as a percentage of that of an equimolecular quantity of acetylcholine. Each of these figures represents a comparison in which the substance tested and acetylcholine were given in alternate doses until doses were found which produced equivalent effects. We are indebted to Dr H. W. Dudley for specimens of most of these esters, and to Messrs Merck for the specimen of carbaminoylcholine.

Sometimes the effects produced could not be satisfactorily compared, because the two substances described curves of quite different shapes on the drum. The figure obtained in such cases depends on the length of time the drugs are left in contact with the tissue, but usually the ratio measured in this way was approximately constant in any one experiment. In some cases the figure obtained was confirmed in a second experiment, but the exact result probably varies with uncontrolled alterations in the conditions of the experiment. Matthes [1930] found that the activity

of pyruvylcholine on the rabbit's intestine was 23 p.c. of that of acetylcholine. In our experiments the figure was 14 p.c. Variations of this kind are probably inevitable, and the figures given in Table I cannot be expected to be always accurately true. These considerations do not detract from, but tend rather to enhance, the value of parallel quantitative tests, as a means of differentiating between acetylcholine and other esters.

All of the esters given in Table I, except carbaminoylcholine, are active unstable substances whose effects on the rectus are increased by eserine to about the same extent as those of acetylcholine, and the first five of the special tests given above would probably not differentiate

TABLE I. Showing the relative potency per molecule, of choline and some of its esters measured by different tests in comparison with acetylcholine, the potency of which is taken as 100 in each case.

Substance	Rabbit		Frog's rectus abdominis		Leech (after eserine)
	Intestine	Blood-pressure (depressor effect)	Normal	After eserine	
Choline	0.075	0.005	0.14	0.035	0.015
Propionylcholine	3.000	4.000	550.00	450.000	45.000
Butyrylcholine	0.240	0	90.00	115.000	90.000
Valerylcholine	0.200	0	25.00	30.000	0.900
Glycylcholine	0.220	0.250	1.20	1.000	0.130
Pyruvylcholine	14.000	10.000	13.00	13.000	16.000
Carbaminoylcholine	80.000	15.000	18.00	5.000	12.000

them. If, however, an extract containing one of these substances was tested in several different ways, there would in most cases be no difficulty in showing that its activity was not due to acetylcholine. Pyruvylcholine is exceptional in that the ratio of its activity to that of acetylcholine appears to be fairly constant, and it would probably be difficult to differentiate it by biological tests.

We have not been able to obtain a satisfactory estimate of the activity of many of these substances on the normal leech, because the preparation is very insensitive until it has been treated with eserine, and the quantities of the esters available were limited. The preparation was sensitized by eserine to all these substances except choline. In most cases the sensitization was very marked, but in confirmation of Minz's results [1932] we found that carbaminoylcholine was quite active on the normal leech (about six times as active as acetylcholine), and its action was comparatively little increased by eserine. As Minz points out, these facts are probably due to the fact that carbaminoylcholine is not readily attacked by the esterase present in the tissue.

The potency of choline on the normal leech was found to be about a quarter of that of acetylcholine, so that this preparation would be useless in testing for acetylcholine in tissue extracts, which always contain a large excess of choline. When treated with eserine, on the other hand, the leech forms a valuable test object, which can be used in parallel with the frog's rectus to test crude extracts.

The results of some of our experiments on the rabbit's blood-pressure confirm some results obtained by Simonart [1932] in experiments on the cat's blood-pressure. Simonart found that propionylcholine was a less active vaso-dilator than acetylcholine, and that butyrylcholine was less active still, but after atropine, when all three substances produced pressor effects, the order of their activities was reversed. In our experiments on rabbits the contrast between the dilator activities of these three substances was in the same direction, but more marked. Butyrylcholine showed no real depressor action because the initial small fall in blood-pressure which it produced was immediately complicated by a secondary pressor action, even in the absence of atropine. Valerylcholine was very similar to butyrylcholine in these respects.

Our results with carbaminoylcholine on the rabbit's blood-pressure do not confirm those obtained by Kreitmair [1931] and Nöll [1932], using cats. Nöll injected carbaminoylcholine into a spinal cat whose blood-pressure was raised by the slow infusion of adrenaline, and found carbaminoylcholine to be about 1000 times as active as acetylcholine. In order to test the possibility that this discrepancy might be due to the use of a different technique, we have repeated Nöll's experiment, using a spinal cat and infusing adrenaline at about the same rate. In this experiment the activity of carbaminoylcholine was about 40 p.c. of that of acetylcholine. We are unable to explain the discrepancy. We have obtained similar results in several experiments. We have been in correspondence with Dr Kreitmair and, in spite of his friendly assistance, have been unable to find any explanation of the fact that we consistently find carbaminoylcholine to be less active than acetylcholine on the blood-pressure of the cat and rabbit, while he finds it to be more active.

#### THE APPARENT ACETYLCHOLINE-CONTENT (A.C.-EQUIVALENT) OF SOME TISSUES.

Extracts have been made of a large number of tissues by the method described above, and these have been compared with known solutions of acetylcholine chloride by means of the eserinated frog's rectus. The

acetylcholine equivalents, or apparent contents of acetylcholine calculated as the chloride are shown in Table II.

TABLE II. A.C.-equivalents (apparent acetylcholine chloride contents) of some tissue extracts.

Tissue	Animal	A.C.-equivalent ( $\gamma$ per g. of tissue)
Spleen	Horse, Ox	4-30
Placenta	Human	28
Submaxillary gland	Dog	1.9, 1.25, 1.5, 3.3, 1.05, 3.3, 2.5, etc.
—	Cat	1.5
Small intestine muscle	Dog	1.8
Small intestine mucous membrane	Dog	1.7
Small intestine	Rabbit	4, 2.8
—	Horse	2.0, 1.8
Large intestine	Dog	2.0
Sympathetic nerve	Horse	1.3, 3.3, 3.9
Auricle	Dog	1.2, 1.3, 0.93, 1.9
Bladder	Dog	1.2, 1.2
Stomach	Rabbit	1.1, 1.3
—	Dog	0.7
Parotid gland	Cat	0.9
Suprarenal gland	Horse	0.6, 0.4
Pancreas	Horse	0.6
Eye	Rabbit	0.6
Fœtus	Rabbit	0.45
Œsophagus	Cat	0.4
Uterus	Rabbit	0.4
Brain		0.4
Vagina		0.3
Ventricle		0.1, 0.07, 0.21, 0.35
Trachea	Dog	0.2
Pancreas		0.13, 0.22
Lung		0.13
Liver		0.11
Skin	Rabbit	0.1
Skeletal muscle	Horse	0.08, 0.08, 0.06
—	Rabbit	0.06
Blood	Horse	0.05, 0.065
—	Dog	0.05, 0.05
—	Ox	0.08, 0.08, 0.06
Plasma	Horse	Nil (<0.015)
Placenta	Rabbit	Nil (<0.1)
Fat		
Testis		
Kidney		
Diaphragm		
Mesentery		
Spleen	Dog	Nil (<0.1)

In every case in which a figure is given for the A.C.-equivalent the effect was due to some substance, the action of which was increased by treating the preparation with eserine. In the case of those extracts in which no figure is given we can say with confidence that there cannot have been as much as 0.1 $\gamma$  of acetylcholine per g. of tissue. Quantities smaller than this cannot always be detected with certainty by this

method because it is necessary to use very highly concentrated extracts, and when crude concentrated extracts are made from some tissues they contain sufficient potassium to interfere with the test.

In some respects these results conform with expectation. The tissues with the highest A.C.-equivalent are mostly tissues whose main activity is controlled by nerves which may be supposed to act by liberating acetylcholine, but there are certain unexplained anomalies.

### *Blood.*

A separate discussion is published [Dudley, 1933] of the attempts that have been made to account for the fact that the results obtained in this laboratory fail to confirm the observation of Kapfhammer and Bischoff [1930], that normal blood may contain large quantities of acetylcholine. By means of the frog's rectus it has been possible to detect an acetylcholine-like activity of a very low order, corresponding to a fraction of 1 p.c. of the quantities found by the Freiburg workers. Although the other tests used would have been sufficiently sensitive to detect quantities of acetylcholine considerably smaller than those stated to be sometimes present, they were less specific than the test on the frog's rectus and failed to detect any activity which could, with certainty, be attributed to acetylcholine. The following account is confined to a description of the results obtained with the frog's rectus.

In one experiment 200 c.c. of citrated jugular blood from a horse were centrifuged, and the plasma was poured off. A concentrated extract of this plasma was prepared by the method recommended above, using trichloroacetic acid. No activity could be detected in this extract, though the concentration of the solutions used was such that as little as 0.015 $\gamma$  of acetylcholine per c.c. of original blood would have produced an effect if it had been present.

A similar extract of the blood cells, also prepared by simple precipitation with trichloroacetic acid, appeared at first to contain about 0.4 $\gamma$  of A.C.-equivalent per c.c. of blood, but the rectus was not sensitized to this extract by eserine, and the effect was probably largely due to potassium. Twenty volumes of absolute alcohol were added to the extract, the precipitate was filtered off and the alcohol removed *in vacuo*. The contraction of the rectus which the extract now produced was increased in the presence of eserine, though to a rather smaller extent than that due to acetylcholine, and the activity was equivalent to 0.065 $\gamma$  of acetylcholine per c.c. of blood. Three extracts of ox blood prepared by Dr Dudley and purified according to Kapfhammer and Bischoff's

method by means of Reinecke salt were also tested on the frog's rectus and contained activity corresponding to about 0.09, 0.08 and 0.06 $\gamma$  acetylcholine per c.c. of blood. A record of the effect obtained in one of these experiments is shown in Fig. 7. It will be seen that, in this case also, the effect was increased by eserine, but not to the same extent as that of acetylcholine. This may be due either to the fact that the substance in blood responsible for these effects was not acetylcholine, or that it was acetylcholine mixed with sufficient choline, or other substances, to affect its action. The aurichlorides prepared from these

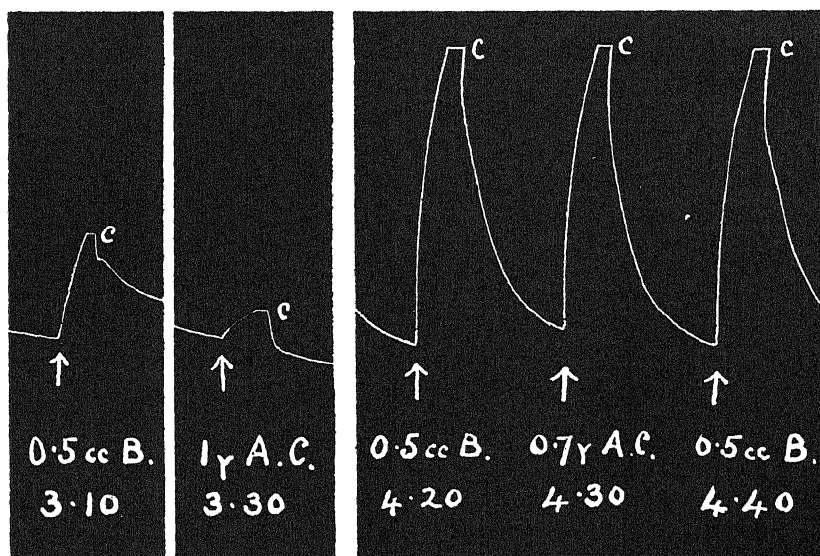


Fig. 7. Frog's rectus abdominis. Eserine sulphate ( $10^{-5}$ ) added at 3.40 p.m. B. Purified blood extract (see text) concentrated 16 times. A.C. Acetylcholine.

extracts also showed activity of the same kind. These results are very different from those reported by Bischoff, Grab and Kapfhammer [1931] who found in some cases as much as 40 $\gamma$  of acetylcholine per c.c. of blood.

The small quantity of A.C.-equivalent which we were able to detect in blood was removed by centrifuging, and was therefore bound in some way with the blood cells. It has been shown that, after a dose of eserine, acetylcholine or some similar substance may be present in the blood in the submaxillary veins [Babkin, Stavratsky and Alley, 1932], and in

the portal veins [Feldberg and Rosenfeld, 1933]. In both cases the active substance must have been in the plasma. The concentrations detected in these experiments were of the same order as the concentrations which we have been able to detect in normal blood by extraction with trichloroacetic acid and alcohol.

#### *Voluntary muscle.*

In the experiments of Bischoff, Grab and Kapfhammer [1932] an extract of voluntary muscle from an ox contained as much as 194 $\gamma$  of acetylcholine per g. of tissue. We have not tried, as in the experiments with blood, to imitate every detail of the experiments of these workers, but we have prepared ten extracts by different methods, suitable for the conservative extraction of acetylcholine if present, from the muscles of the horse, dog, cat, rabbit and frog, and have never obtained evidence of more than 0.08 $\gamma$  of A.C.-equivalent per g. of tissue. In this respect our results are similar to those of Plattner and Krannich [1932].

#### *Spleen.*

Dale and Dudley [1929] drew attention to the contrast between the large amount of acetylcholine contained in the spleens of horses and oxen, and its apparent absence from the spleens of dogs and other smaller animals. The reality of this contrast has now been confirmed by a more specific test. The extracts of dog's spleen did not contain as much as 0.1 $\gamma$  of A.C.-equivalent per g. of tissue.

#### *Placenta.*

We have been able to confirm the observation of Bischoff *et al.* [1932] that extracts of human placenta contain very large quantities of a substance pharmacologically identified as acetylcholine (28 $\gamma$  per g. of tissue). The extract was prepared with alcohol and compared with acetylcholine for its action on a piece of rabbit's intestine, and on the frog's rectus abdominis. The results obtained in these two comparisons agreed quantitatively with one another. On the other hand an extract of rabbit's placenta contained no detectable acetylcholine.

The presence of these large quantities of acetylcholine in the human placenta is as difficult to relate to any known physiological function as that of similar quantities in the spleens of large ungulates. In neither case is it yet possible to offer any explanation of the presence of a high concentration of acetylcholine, limited to certain organs of certain species. We have not discovered any similar anomalies among the other extracts we have tested.



*Suprarenal gland.*

The results of the tests of extracts of suprarenal gland are of rather special interest because the original investigation of the pharmacological properties of acetylcholine was undertaken by Hunt and Taveau [1909] because they had found that the vaso-dilator effects of extracts of suprarenal from which the adrenaline had been removed could not be entirely due to choline, and they believed that some simple derivative of choline might be partly responsible for the effects observed. Our conclusion that suprarenals contain a substance pharmacologically identified as acetylcholine confirms the result of Minz's experiments with leeches [1932].

*Sympathetic nerves.*

The observation that extracts of a horse's sympathetic nerve appeared to contain large quantities of acetylcholine was surprising. In one experiment the estimate obtained on a frog's rectus was quantitatively confirmed in an experiment with an eserinizied leech. In two other experiments attempts were made to discover whether the activity was more concentrated in the ganglia, or in the parts of the sympathetic chain between the ganglia. The ganglia are not very distinct structures in the horse, but the portions of the nerve immediately surrounding the point of entrance of the rami communicantes were dissected out and extracted separately from the rest of the nerve. The estimates of the A.C.-equivalents of the two extracts prepared in this way were, however, identical.

It is known that acetylcholine has a marked action on sympathetic ganglia, and these results therefore suggested the theory that acetylcholine might play a part in the normal transmission of impulses through ganglia. We have obtained no direct evidence in favour of this theory, but the recent work of Kibjakow [1933] seems to support it. Kibjakow has shown that the stimulation of preganglionic fibres in the cat causes the liberation in the superior cervical ganglion of a substance capable of acting on ganglion cells. Our results suggest that this substance may be acetylcholine.

*Heart.*

The observation that extracts of mammalian auricles contain much larger quantities of A.C.-equivalent than extracts of ventricles confirms the conclusions of other investigators. Jendrassik [1923] found that watery extracts of rabbit's auricle stimulated an isolated rabbit's intestine while extracts of ventricle produced pure inhibition. Engelhart [1930],

working in conjunction with Plattner, found that the normal A.C.-equivalent of an alcoholic extract of rabbit's auricle, as measured on a frog's heart, was about  $4\gamma$  per g., while that of the ventricle was only  $0.13\gamma$  per g. These figures are of the same order as those recorded in Table II. This difference between the auricles and ventricles was foreseen by Engelhart on the grounds that the vagus has little or no direct action on the ventricles, but only affects them as a secondary consequence of its action on the auricles [see Dale, A. S., 1930].

### *Intestine.*

The results obtained with extracts of intestine had also been foreshadowed. The intestine contains large quantities of choline. Choline has a powerful stimulant action on the isolated intestine, and acetylcholine has the same action in about one-thousandth of the concentration. Le Heux [1919] found that the salts of various acids which form active choline esters also stimulated the rabbit's intestine when added to the bath in which a piece of isolated intestine was suspended, and that this action was antagonized by atropine. He therefore suggested that the intestine was able to synthesize choline esters under these conditions. This conclusion was confirmed by much other evidence.

More recently Feldberg and Rosenfeld [1933] have found that the portal blood in an eserinated dog or monkey differs from systemic blood in containing a substance, pharmacologically identified as acetylcholine. This substance did not occur in the blood in the splenic vein and must have been derived from the intestine.

It was therefore probable that a choline ester would be found to occur naturally in extracts of intestine. Evidence on this point cannot be obtained by testing crude extracts, except when a highly specific pharmacological test such as that on the rectus or leech is used, because those extracts contain not only potassium, histamine and choline, but also adenosine compounds and the substance *P* [Euler and Gaddum, 1931].

In order to confirm the conclusions reached from the experiments with the rectus, an extract was therefore purified by chemical methods calculated to remove these other active substances. This extract was originally prepared for other purposes from horse's intestine without all the precautions that might have been applied to avoid partial hydrolysis of choline esters. An unknown loss therefore probably occurred before the first test was made, but the extract contained a substance unstable in alkali, and sufficient activity was present to enable the purification to be carried further. The original extract was in acid alcohol. This was taken

down *in vacuo* at 40° to small bulk and defatted with ether. A large quantity of absolute alcohol was then added and the precipitate, which was inactive on the frog's rectus, but which contained practically all of the substance acting like adenosine, was thrown away. The process was repeated until the extract could be taken down to dryness, and what was left of the activity (about 40 p.c.) was then dissolved in absolute alcohol. Platinum chloride was added. The filtrate from this contained no acetylcholine, and all that was left (about 22 p.c.) of the substance acting on the frog's rectus was in the precipitate.

The base was liberated from the chloroplatinate by means of metallic silver [Dudley, 1929], and was tested on the frog's rectus. The chloroplatinate was thus found to contain base corresponding in activity to 0.115 p.c. of acetylcholine chloroplatinate. The extract was acetylated, and again tested on the rabbit's intestine. From this result it would be calculated that the precipitate contained 8.4 p.c. of choline chloroplatinate, so that only about 1.4 p.c. of the total choline in the precipitate was apparently in the form of acetylerster. The effect of the solution prepared from the platinum precipitate on the normal rabbit's intestine was indistinguishable from the effect of the amount of acetylcholine, which it had been estimated by means of the frog's rectus to contain, but when atropine was added, the extract, when given in large doses, still had an action—presumably due to the substance *P* [Euler and Gaddum, 1931]. This substance was almost completely removed by washing the platinum precipitate repeatedly with absolute alcohol—a procedure which reduced its weight by 27 p.c. without any detectable loss of the substance acting on the frog's rectus. The final precipitate was free of adenosine compounds and substance *P*, but presumably still contained histamine and large quantities of choline. It was tested on the rabbit's intestine and in the rabbit's blood-pressure and also by injection into the artery leading to a cat's gastrocnemius, which had been rendered sensitive to acetylcholine by denervation a fortnight before the experiment. The four estimates of the amount of acetylcholine in this precipitate are given in Table III.

The agreement between these results was considered satisfactory. The actions of this extract, like those of acetylcholine, on the rabbit's

TABLE III. Estimates of the A.C.-equivalent of a purified precipitate from horse's small intestine. Parts per thousand.

Frog's rectus	0.90
Cat's gastrocnemius	0.75
Rabbit's intestine	0.80
Rabbit's blood-pressure	0.84

intestine and blood-pressure were abolished by atropine, while that on the cat's gastrocnemius was not. The extract was treated with normal blood and with eserinized blood, and then deproteinized and tested on the frog's rectus and rabbit's intestine. Both types of test showed that the active substance was destroyed by blood and that the destruction was inhibited by eserine. This extract was not tested on the eserinized leech, or on the eserinized frog's rectus, because it was prepared at an early stage of the investigation before these simple tests were known; but, nevertheless, the experiments which were performed provide strong evidence for the identification of the substance in extracts of intestine, which is mainly responsible for the action on the frog's rectus, as acetylcholine. Pyruvylcholine is the only other substance in Table I which could have been responsible for these effects.

#### *Other extracts.*

Emphasis must be laid on the fact that in most other cases the only special test which was applied consisted in the observation that the action on the rectus was increased by eserine, so that the effect observed might have been produced by almost any choline ester. Indeed, the effects of extracts of dog's bladder differed sufficiently in their time relations from those of acetylcholine to suggest that they may really be due to some other choline ester. An extract of testis contained some substance which produced a contraction of the rectus similar to that caused by acetylcholine, and equivalent to 0.4 $\gamma$  of acetylcholine per g.; but as this effect was not increased by eserine it cannot have been due to acetylcholine, or to any choline ester likely to occur in a tissue extract, and this figure is not included in Table II. This phenomenon, which was observed twice, was not observed with extracts of other tissues.

The distribution of A.C.-equivalent shown in Table II is in some respects similar to the distribution of choline esterase shown by the experiments of Plattner and Hintner [1930]. Thus extracts of intestine, salivary glands, suprarenal, brain and uterus contained more than 0.3 $\gamma$  of A.C.-equivalent per g., and sufficient esterase to halve the concentration of acetylcholine in less than 10 min. under the conditions of experiment. Lung, ventricle, skeletal muscle and kidney contained less than these quantities of A.C.-equivalent and of esterase. On the other hand, blood and dog's pancreas, liver and spleen, failed to comply with this classification. These tissues were all rich in esterase and poor in A.C.-equivalent. There were no tissues rich in A.C.-equivalent and poor in esterase.

## FACTORS AFFECTING THE A.C.-EQUIVALENT OF TISSUES.

The most generally successful method of demonstrating the liberation of substances by nerves has consisted in stimulating the nerves to tissues bathed in salt solution, and then detecting the appearance of pharmacological activity in the salt solution. Experiments of this kind provide no evidence as to whether the active substance is actually formed in response to the stimulus or merely liberated from a pre-formed store. Evidence on this point can be obtained by measuring the A.C.-equivalent of tissue extracts before and after stimulation of the nerves. Experiments of this kind have been carried out on the heart. Witanowski [1925] showed that stimulation of the frog's vagus increased the A.C.-equivalent of the alcoholic extracts of the frog's heart. Plattner [1926] found that the same phenomenon could be demonstrated in the ventricles of rabbits, dogs and cats. Englehart [1930] confirmed these observations, but emphasized the fact that in mammals the auricles contain very much more A.C.-equivalent than the ventricles, and that the largest increase occurs in the auricles.

We have attempted to carry out similar experiments with the submaxillary glands, but have not succeeded in showing any marked and consistent change in the A.C.-equivalent of extracts when the chorda tympani was stimulated.

In these experiments the dogs were anaesthetized with ether followed by chloralose. The chorda (nervus submaxillaris) was stimulated on one side for 30-60 min. and 15-20 c.c. of saliva were secreted. The glands were then removed and immediately minced under the surface of 10 p.c. trichloroacetic acid. The extracts were treated as described above and tested on the eserinated frog's rectus. Within these limits the details of the procedure were varied in the different experiments in the hope that conditions would be found under which the stimulation would produce some recognizable change in the A.C.-equivalent of the gland. The results are recorded in Table IV. In the last of the experiments recorded in this table the dog had received an intravenous injection of 0.2 mg. of eserine

TABLE IV. Showing the A.C.-equivalent of dogs' submaxillary glands. Normal resting glands compared with those removed after stimulation of the chorda.

$\gamma$ per g. of wet tissue		$\gamma$ per gland	
Resting	Active	Resting	Active
4.0	4.0	20	19
1.3	1.5	11	12
1.2	1.1	8	10
2.5	2.5	14	17

sulphate per kg. There was no evidence that the A.C.-equivalent was affected by stimulation of the nerve in any of these experiments.

Henderson and Roepke [1933] have made similar experiments, and reached similar conclusions. These results are recorded here because Beznak [1932] has obtained very different results. His figures for the total A.C.-equivalent of normal glands mostly lie between 0.2 and 0.4  $\gamma$ , and are thus much lower than ours. In some of his experiments extracts of active glands had an action on the frog's heart corresponding to ten times as much acetylcholine as the extracts of resting glands, but his highest figure for an active gland is still lower than our lowest figure for a resting gland. The reasons for these discrepancies are still uncertain, but we believe that they are connected with the fact that the frog's heart is affected by a large number of substances in tissue extracts besides acetylcholine, and that some of these substances are antagonistic to acetylcholine.

We have also studied the effect, on the A.C.-equivalent of extracts of salivary glands, of the degenerative section of parasympathetic nerves. The chorda was divided and a portion of the chorda-lingual nerve was removed with aseptic precautions in seven dogs. At intervals varying from 24 hours to 69 days later the dogs were anaesthetized with ether and the submaxillary glands were exposed. The two glands were removed almost simultaneously and immediately placed each in a weighed beaker containing 10 p.c. trichloroacetic acid, cut up with scissors, and weighed. The extract was then prepared in the usual way and tested against acetylcholine on the frog's rectus in the presence of eserine. The results of these experiments are shown in Table V.

TABLE V. Showing the A.C.-equivalent of dogs' submaxillary glands. Normal glands compared with those removed after degenerative section of the chorda.

Days after section of chorda	$\gamma$ per g. of wet tissue		$\gamma$ per gland	
	Normal	Denervated	Normal	Denervated
1	1.90	0.90	11	5
6	1.25	1.25	12	9
13	1.50	0.75	11	5
14	3.30	2.40	28	19
17	1.00	1.00	11	6
44	3.30	1.30	22	7
69	2.50	1.60	28	14
Controls:				
Sympathectomy 13 days previously	4.1	4.1	25	26
Both glands normal	2.8 3.0	— —	13 14	— —

In confirmation of earlier work [Langley, 1885; Bradford, 1888] we found that this operation caused a loss of weight of the gland. The mean loss was  $18 \text{ p.c.} \pm 5$  (S.D. of mean). In every case the whole denervated gland contained definitely less A.C.-equivalent than the whole normal gland. In every case except two, the concentration was diminished. In the two exceptional cases, although an absolute loss of A.C.-equivalent occurred, the concentration of activity in the gland was apparently unaffected. In a control experiment in which both glands were normal, no significant difference in A.C.-equivalent could be detected. In another experiment the sympathetic nerve supply to the gland was interrupted by excision of the superior cervical ganglion. Extracts of both glands were prepared 13 days later, and again no significant difference in A.C.-equivalent could be detected.

It is obvious, without calculation, that the section of the chorda caused a significant fall in the total A.C.-equivalent of the gland, but it is not obvious whether it had a significant effect on the A.C.-equivalent per g. The percentage fall of A.C.-equivalent per g. in each experiment was therefore calculated. The mean of these values was 33 p.c., and the probability obtained from Student's table of  $t$ , that this represented a significant effect was between 0.98 and 0.99 [see Fisher, 1925]. There is thus very little doubt that denervation diminished not only the total A.C.-equivalent of the gland, but also its concentration.

The change involved the loss of about half the activity and did not appear to be progressive after the first 24 hours. Many of the nerve cells in the course of the secretory fibres of the chorda tympani lie in the submaxillary ganglion at the hilum of the gland, but others are scattered along the course of the nerve [Langley, 1898], so that it is probable that in our experiments some preganglionic and some postganglionic fibres were divided. In order to discover whether section of these two classes of nerve fibre would produce different effects, we have carried out some experiments on the parotid glands. The nerve cells in the course of the secretory fibres to the parotid gland are believed to lie in the otic ganglion, and these fibres can therefore be divided peripherally to the ganglion. These experiments were carried out simultaneously with two of the experiments recorded in Table V. The branch of the auriculo-temporal nerve which joins the branch of the facial nerve on the lateral surface of the masseter, was traced centrally to beyond the point where the nerve to the parotid gland leaves it, and excised: 13 and 17 days later the parotid glands were dissected out and extracted in the same way as the submaxillary glands. It was not found possible to remove these glands so

completely as to obtain a reliable estimate of their total A.C.-equivalent, and we contented ourselves with a comparison of the concentrations. In the first dog the concentrations of A.C.-equivalent in the normal and denervated glands were 0.6 and 0.24 $\gamma$  per g., and in the second dog the corresponding figures were 0.3 and 0.12 $\gamma$ . In each case, therefore, there was a loss of about 60 p.c. of the A.C.-equivalent per g. of gland. This loss is not significantly different from that observed in submaxillary glands, so that these experiments have failed to prove that there is any difference in the reaction of salivary glands to the degenerative section of pre-ganglionic and postganglionic nerve fibres.

Again our results stand in contrast to those of Beznak [1932] who observed no change in the A.C.-equivalent of the gland as a result of degenerative section of the nerves.

#### DISCUSSION.

Acetylcholine cannot be present as such, freely diffusible in tissues, in the quantities suggested by the pharmacological determination of the A.C.-equivalent of extracts. In the first place the concentration present would be more than sufficient to produce large effects on many of the tissues concerned. In the second place, the acetylcholine would be rapidly destroyed by choline esterase unless it were protected from this enzyme in some way.

In explanation of these facts, it has been suggested that the substance whose concentration is measured in experiments of this kind is not a choline ester at all, but some inactive precursor which is not attacked by the enzyme. An alternative theory is that the choline ester is present as such, but is stored in some special structure which prevents it from diffusing freely through the tissue. The fact that mincing the tissue leads to the disappearance of A.C.-equivalent favours this second theory, but whether this is true or not, the question of whether the methods of extraction used are liable to cause the formation of choline esters from some inactive precursor cannot be decided at present. We do not yet know how choline esters are formed in tissues.

The question as to whether the stimulation of the nerves causes the new formation of A.C.-substance, or merely liberates it, has already been mentioned. In the former case it might be expected that, after stimulation, the A.C.-equivalent would increase; in the latter case it should be diminished. There is some evidence, discussed above, of an increase of the A.C.-equivalent of the heart as the result of stimulating the vagus.



In the case of the submaxillary glands, there is as yet no conclusive evidence either way. Gibbs and Szelöczy [1932 *b*] found that stimulation for 30 sec. of the nerve to a dog's submaxillary gland, perfused with salt solution, caused the appearance of 0.02–0.05 $\gamma$  of A.C.-equivalent in the perfusion fluid. On re-injection this was sufficient to cause the secretion of about half the quantity of saliva originally secreted as a result of stimulating the nerve, and it is therefore probable that the total amount originally liberated in half a minute was not more than 0.1 $\gamma$ . The results given above show that the total amount normally extractable from the gland is 10–30 $\gamma$ , and this would clearly be sufficient to keep the perfused gland active for a considerable time. If these results are applicable when the blood supply to the glands is normal, it is clear that no very marked change in the A.C.-equivalent in either direction can be expected as the result of stimulating the nerve for periods of less than half an hour. In the experiments described above stimulation was continued for this period, and longer, without effect. This is not really surprising, since it is quite possible that the A.C.-substance was replaced as rapidly as it was liberated.

Similar arguments may be applied to the heart. In an experiment of Witanowski's [1925] an extract corresponding to only 1.3 p.c. of a single heart produced a very striking effect when applied to another heart. The fact that, even when the nerve has not been stimulated, these tissues contain such large quantities of extractable A.C.-substance tempts us to put forward the teleological argument that it is easier to suppose that the direct effect of stimulation of the nerves is the liberation of the active substance already present, rather than the addition of fresh activity to an already ample store.

In the experiments shown in Table V, the A.C.-equivalent of the whole gland was approximately halved as the result of degenerative section of the chorda. This might be due to the degeneration of the hypothetical structure which contains the A.C.-substance, but if this is so, the effect is surprisingly rapid in appearance and surprisingly incomplete. It is also possible that the A.C.-equivalent is normally kept up by nerve impulses passing down the chorda, and that when these impulses are cut off, the production of A.C.-substance still continues, but at a slower rate. In any case these results show that the paralytic secretion which occurs after section of the chorda is not due to an excess of A.C.-substance in the gland.

## SUMMARY.

1. Pharmacological methods for detecting choline esters in tissue extracts are discussed. The most satisfactory test objects are the frog's rectus abdominis and the longitudinal muscle of the leech.

2. Many choline esters can only be distinguished pharmacologically from one another by carrying out several assays by different methods and comparing the results quantitatively with one another.

3. An unidentified substance (*R*) occurs in some extracts, which produces an effect on the isolated frog's heart superficially similar to that of acetylcholine, but not affected by atropine.

4. The apparent acetylcholine chloride contents (A.C.-equivalents) of a number of tissues have been measured. The finding by Kapfhammer and his co-workers of large quantities of acetylcholine in blood and other tissues has been confirmed only as regards the human placenta. In other cases the amounts detected have been comparatively small. In this respect our conclusions agree with those of most other workers.

5. Degenerative section of the parasympathetic nerves to salivary glands causes the disappearance of about half of their A.C.-equivalent in 24 hours.

6. Methods of confirming the pharmacological identification of acetylcholine are discussed. There is no reason to doubt that most of the extracts which we prepared did actually contain acetylcholine.

It is a pleasure to acknowledge the help which Dr H. W. Dudley has given us in this investigation.

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## INSULIN AND ADRENALINE.

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THE suggestion has been put forward recently that certain effects following the administration of insulin to the intact animal might be due to the secondary secretion of adrenaline (Cori [1931], Corkill [1930]). Whilst the evidence appears good that the secretion of adrenaline is stimulated when the blood sugar falls to hypoglycæmic levels, such a view would suggest almost as a corollary that certain effects of adrenaline might be due to insulin and that, as far as the intact animal is concerned, an impasse would be reached in elucidating the essential actions of these hormones. These matters become particularly involved when it is found that certain results of the injections of both adrenaline and insulin appear to be identical, at least qualitatively. Thus, both hormones can, under properly controlled conditions, produce an accumulation of glycogen in the liver of the starving young rabbit (Corkill [1930], Sahyun and Luck [1929], Bischoff and Long [1930], Goldblatt [1929]). This effect was found by some workers to be accompanied by a loss of muscle glycogen in both cases.

General agreement has been reached that insulin stimulates the peripheral oxidation of carbohydrate, and there appears to be good evidence that adrenaline depresses it. This latter view is based on the fact that considerable and long-lasting hyperglycæmia can be produced in the presence of small quantities of hepatic glycogen, such hyperglycæmia in the uninjected animal requiring the infusion intravenously of amounts of glucose far in excess of that available in the liver. It is therefore concluded that two factors make persistent adrenaline-hyperglycæmia possible, viz. inhibition of peripheral oxidation and resynthesis of the products of the breakdown of peripheral glycogen when they

<sup>1</sup> In receipt of a part-time grant from the Medical Research Council.

reach the liver, so that, provided no loss occurs by way of the kidneys, there is a cycle of changes which sustains the blood sugar above normal. The claim that certain effects of insulin are due to adrenaline is particularly directed to those effects which do not accord well with the generally accepted view of the action of insulin. There is no doubt that, in the intact animal, the effects obtained at any moment from any treatment with a hormone is necessarily complicated by the simultaneous action of other internal secretions. Even the injection of an excess of one hormone does not remove the difficulty of quantitative interpretations. The conception of homeostasis implies that the internal adjustments in the organism make the intact animal unsuitable for the investigation of the essential action of hormones. Qualitatively, however, much can be obtained by the use of the intact animal. For example, no complication of the action of adrenaline by the secondary secretion of insulin could explain adrenaline hyperglycæmia, nor could the rise in oxygen utilization and R.Q. following the injection of insulin be regarded as due to the simultaneous action of adrenaline. In the majority of animals the effect of insulin is to deplete the hepatic glycogen, even when the animal is actively absorbing sugar. Now insulin without doubt brings about a deposition of glycogen in the liver of the depancreatized or phlorizinized animal, but it would not be suggested that this is due to a secondary secretion of adrenaline. Indeed such secondary secretion might be invoked to explain those cases in which insulin depletes liver glycogen, this hormone being of importance in making the glycogen available for the increased oxidation produced by the insulin. A view which has not received attention is that increased peripheral oxidation might reasonably stimulate the endogenous formation of glycogen in the liver.

The questions which will be considered in this paper are:

- (1) Is the redistribution of glycogen in young rabbits after the injection of insulin similar to that after the injection of adrenaline?
- (2) Is the loss of muscle glycogen after insulin sufficient to account for the increase in liver glycogen which occurs in the same conditions?
- (3) Is the action of insulin in these animals associated with a definite increase in blood lactate, the latter being regarded as evidence of the secondary secretion of adrenaline?
- (4) Can the effect of insulin be dissociated from that of adrenaline by means of ergotamine or iodoacetic acid?

## EXPERIMENTAL.

Glycogen was estimated by a modification of Pflüger's method similar to that described by Lovatt Evans and his co-workers [1931]. The lactate in blood was estimated by the method of Friedmann, Cotonio and Shaffer and blood sugar by Maclean's method.

The animals used in these experiments were mainly young rabbits. The results were compared for litter mates only and, unless otherwise stated, the values for muscle glycogen represent those for mixed samples taken from all four limbs. Where values are given for the total glycogen in the animal, liver and muscles only are meant, the assumption being that the muscles constitute about 50 p.c. of the body weight. All estimations of tissue glycogen are subject to some doubt if referred back to the living animal. The recent findings of Anderson and Macleod [1930] indicate, however, that the rate of loss of glycogen from the muscles post-mortem is very slow if the muscles are uninjured. To test this point in young rabbits they were killed by a sharp blow at the base of the skull, extended and the lumbar spine transected immediately. The gastrocnemius, tibialis anticus and vastus internus were carefully dissected out from the right limb and worked up for glycogen; the animal was left at room temperature (unless otherwise stated in the table) and the corresponding muscles on the left side removed after the times stated in Table I. As Anderson and Macleod found in cats, there is a definite discrepancy between the glycogen of the muscles both on the same side and on opposite sides, but the averages are very close. As is seen in Rabbit No. 6, injury to the muscles leads to marked loss in glycogen. Rather a large loss occurred in No. 7 when the limb was kept immersed in saline at 37° C. It is also seen that the effect of adrenaline on muscle glycogen ceases immediately after death. It is clear that reliance may be placed on glycogen values for muscle even up to an hour after death, provided that (1) averages are taken, (2) the muscles are not injured, and (3) the temperature be kept well below body temperature.

As to the loss of liver glycogen after removal of the liver from the body, Lovatt Evans *et al.* [1931] have shown that, when kept at room temperature, the sliced liver loses 5 p.c. of its glycogen in 1 min. and 40 p.c. in about 8 min., after which the rate of loss is very slow. In similar experiments we have found a similar rate. In the experiments in this paper the liver samples were in the hot potash in about a minute after removal from the body, so that it was not considered necessary to correct.

TABLE I. Post-mortem changes in glycogen content of mammalian muscle.

Rabbit No.	Wt. kg.	Right p.c.	Left p.c.	Remarks and time between muscle removal
1	1.26	0.177 0.206 0.190 <u>Av. 0.191</u>	0.165 0.211 0.231 <u>Av. 0.202</u>	1 hour
2	1.50	0.211 0.251 0.185 0.219	0.332 0.208 0.159 0.233	1 hour
3	1.35	0.139 0.114 0.151 0.135	0.093 0.065 0.126 0.095	0.25 mg. adrenaline intravenously 2 min. before death 1 hour
4	1.65	0.202 0.189 0.117 0.169	0.140 0.151 0.175 0.155	As in No. 3
5	1.65	0.368 0.396 0.348 0.371	0.408 0.461 0.457 0.442	0.5 mg. adrenaline intravenously 2 min. before death $\frac{1}{2}$ hour
6	1.00	0.639 0.534 0.397 0.523	0.450 0.394 0.451 0.432	Muscles on left side were bruised 1 hour
7	1.20	0.323 0.262 0.234 0.276	0.250 0.154 0.199 0.201	Left limb kept at 37° C. after death 1 hour

*The effect of adrenaline on glycogen distribution in young rabbits.*

The suitability of these animals for comparative work on glycogen distribution has been demonstrated by the present writer [1929]. It is important that such comparisons be made between litter mates, preferably bred in captivity. All the animals we have used have been so bred. A specially constructed light wooden collar was used during starvation of the animals, to make ingestion of faeces impossible. No difficulty has been found in obtaining animals with empty stomachs in this way if the diet during a day or two before the experimental period was somewhat restricted. In Table II are given the results of subcutaneous injection of adrenaline into these animals after starvation for various periods.

It is seen that in the starving young rabbit there is a considerable uniformity in the total glycogen per unit mass, even after greatly varying

TABLE II. Effect of adrenaline on glycogen distribution in young rabbits.

Litter No.	Time of starvation (days)	Animal No.	Wt. g.	Blood sugar mg./100 c.c.	Glycogen		Total g./100 g. body wt.	Dose and time of action of adrenaline
					Liver p.c.	Muscle p.c.		
1	2	1	587	175	0.55	0.41	0.22	} Control
		2	675	120	0.50	0.36	0.20	
		3	653	360	0.75	0.24	0.14	
		4	640	390	0.26	0.16	0.09	
		5	660	380	0.32	0.35	0.18	
		6	677	360	0.37	0.21	0.11	
2	2	1	785	156	0.31	0.34	0.18	} Control
		2	767	102	0.27	0.27	0.14	
		3	615	340	0.28	0.26	0.14	
		4	775	300	0.60	0.18	0.11	
		5	710	364	0.43	0.15	0.18	
		6	853	330	0.16	0.15	0.08	
3	2	1	495	200	0.52	0.26	0.15	} Control
		2	432	109	0.50	0.26	0.14	
		3	437	41	1.60	0.38	0.25	
		4	485	171	0.29	0.18	0.11	
4	3	1	445	77	0.41	0.36	0.19	Control
		2	408	—	0.15	—	—	0.2 mg. 1½ hours
		3	457	350	0.26	0.19	0.10	1.1 mg. in 4 doses 4¾ hours
5	5	1	550	95	1.30	0.33	0.20	Control
		2	518	264	0.24	0.28	0.15	1.1 mg. in 4 doses 4¾ hours
		3	490	350	0.35	0.27	0.15	As in 2

periods of starvation. The average of the controls was  $0.18 \pm 0.04$  g. per 100 g. animal, whilst the average for the animals injected with adrenaline was  $0.13 \pm 0.05$ . We have introduced in Litter 3 an animal injected with large doses of insulin in order to illustrate the vastly different effect produced in these animals by this hormone. The average weight of these rabbits is about 500 g. and the average loss of glycogen per 100 g. is about 0.05 g., *i.e.* an average absolute loss of about 0.25 g. per animal. There is no reason to believe that this is brought about by increased oxidation or by loss in the urine (very little urine is as a rule excreted in these experiments). We are led, therefore, to the probability that there is a storage of the degradation products of glycogen actually in the tissues after the injection of adrenaline. Whilst the acute effect of adrenaline is a loss of both liver and muscle glycogen, the process of recovery is a peculiar one. It has been known from the time of Claude Bernard that muscle glycogen does not produce glucose but lactic acid.





found that 1 mg. of adrenaline produced a rapid fall in the glycogen of the liver and the muscles, the former recovering and attaining very high values, whilst the latter remained at extremely low levels for as long as 42 hours. This must mean that the lactic acid produced from the muscle lies dormant until such time as the liver is able to work it up to glycogen. If, however, small doses of adrenaline are used it is possible to obtain an almost quantitative redistribution of glycogen from the muscles to the liver in between 2 and 3 hours. This is shown in Table III.

*The effect of a non-glycosuric dose of adrenaline.*

A litter of nine young rabbits was starved for 24 hours. Three were taken as controls, three received 0.15 mg. adrenaline subcutaneously and the remaining three received 1 c.c. pituitrin and 25 min. later 0.15 mg. adrenaline. The last three animals were so injected to see if, as has been stated by some workers, pituitrin exerts an antagonistic effect on adrenaline hyperglycæmia: such an effect was not found.

It is clearly seen that, with this dose of adrenaline, there was no loss in glycogen, a quantitative redistribution having occurred (Table III).

*Time factor in the resynthesis of liver glycogen.*

The time factor in the glycogen redistribution is shown in Table IV, where it is seen that the fall in muscle glycogen after 0.5 mg. adrenaline had reached a maximum in about 1½ hours and from that time on the

TABLE IV. Litter of five young rabbits. 48 hours' starvation before injection.

Rabbit No.	Wt. g.	Blood sugar mg./100 c.c.	Glycogen			Dose of adrenaline and time of action	
			Liver p.c.	Muscle p.c.	Total g./100 g. body wt.		
1	942	380	0.51	0.28	0.16	0.5 mg.	1½ hours
2	985	139	2.87	0.23	0.20	"	4½ "
3	865	125	2.59	0.29	0.24	"	12 "
4	980	129	1.14	0.28	0.18	"	18 "
5	655	152	2.53	0.35	0.26	"	24 "

products of the breakdown of glycogen were being resynthesized in the liver, the rate of glycogenolysis in the liver having become normal in about 4½ hours.

*Summary of experiments on the effect of adrenaline.*

We find that the end-effect of adrenaline depends on the dose and the time of action. With a large enough dose the effect in young rabbits even up to 3 hours after the injection may be loss of glycogen from both

the liver and muscles, but after this time the liver glycogen recovers and far exceeds the initial value although the muscle glycogen may fall no further. This is clearly due to a lag between the liberation of the products of the breakdown of muscle glycogen and their resynthesis in the liver. With a suitable smaller dose of adrenaline the glycogenolytic effect in the liver may be so slight that resynthesis proceeds so rapidly that quantitative redistribution is attained in a relatively short time. This would involve the resynthesis of the glucose liberated from the liver when it reaches the muscles, an effect which is usually associated with the action of insulin.

We proceed now to present results illustrating the effects of insulin in these animals and to examine how far it is justifiable to consider that adrenaline, secondarily secreted, is responsible for them.

*The action of insulin in young rabbits.*

The injection of insulin is followed by an increase in the peripheral oxidation of carbohydrate and in the majority of normal animals this leads to a loss in both muscle and liver glycogen. Corkill [1930] found this to be the case in several different species. To his list of animals we may add the guinea-pig with which the results in Table V were obtained. Ten small guinea-pigs were starved for 24 hours. The muscles used for glycogen estimation were the tibialis anticus and the gastrocnemius; usually about 2 g. could be obtained.

The results shown in Table V are puzzling. The effectiveness of the insulin is clear from the blood-sugar values. No significant change in the

TABLE V. Young guinea-pigs starved 24 hours.

Rabbit No.	Wt. g.	Blood sugar mg./100 c.c.	Glycogen		Dose of insulin and time of action
			Liver p.c.	Muscle p.c.	
1	214	135	0.440	0.619	Controls
2	258	144	0.366	0.619	
3	600	97	0.312	0.552	
4	550	85	0.292	0.631	
5	350	58	0.509	0.310	
6	183	47	0.457	0.412	1 unit 3 hours
7	287	27	1.074	0.377	1½ " 3½ "
8	550	35	0.365	0.770	1½ " 2½ "
9	550	60	0.396	0.559	1 " 3½ "
10	400	0	0.412	0.681	2 " 2½ "
Averages: Controls ...			0.384	0.546	
Treated animals			0.408	0.559	

(Anomalous liver value for No. 7 omitted in average.)

liver or muscle glycogen was found in spite of a fall in average blood sugar, from 104 to 34. Further study of this species will be necessary.

It is, however, with the young rabbit that the striking and apparently paradoxical action of insulin is observed, viz. a very marked increase in liver glycogen, which exceeds any loss which may simultaneously occur in the muscles. In Table VI are shown the results in a typical experiment. Calculated in the usual way, the average total glycogen per 100 g. body

TABLE VI. A litter of eight young rabbits starved for 24 hours.  
The dose of insulin was 1 unit injected subcutaneously.

Rabbit No.	Wt. g.	Blood		Glycogen			Time of action
		Sugar mg./100 c.c.	Lactate mg./100 c.c.	Liver p.c.	Muscle p.c.	Total g./100 g. body wt.	
1	800	168	44	0.549	0.202	0.118	Controls
2	625	156	37	0.552	0.198	0.116	
3	700	143	53	0.613	0.181	0.110	
4	825	88	63	1.804	0.130	0.130	1 50
5	750	106	50	2.647	0.139	0.150	2 10
6	800	84	68	5.042	0.141	0.245	2 55
7	775	96	44	2.824	0.129	0.162	3 15
8	725	106	68	3.426	0.098	0.166	3 30

weight for the controls in Table VI is 0.115 g. and for the injected animals 0.171 g. Certain workers, confirming the increase in liver glycogen here demonstrated, attribute it to the secondary secretion of adrenaline. It will be seen in Table VI that the blood sugar of the injected animals was at an average normal level, this being due to the fact that this litter was of the kind, sometimes met with, in which the blood sugar is high. Can we on the basis of the blood sugar consider that adrenaline was being secreted at a rate sufficient to account for the liver glycogen? In the dog the secondary secretion of adrenaline occurs according to Houssay, Lewis and Molinelli [1924] at a blood-sugar level of 0.05 p.c., and according to LaBarre and Houssa [1932] at 0.075 p.c. The rabbit does not lend itself to determinations of this sort, but it is improbable that the critical level is as high as that of the injected animals in this experiment. On the other hand, the average blood lactate rose from 45 to 59, which might be regarded as slight evidence of an increased secretion of adrenaline; but Matakas [1932], in a recent paper, reports that the rabbit is not reliable in this connection. Averages from young litter mates are, in our experience, reasonably reliable.

The decisive experiment would be to observe the effect of insulin in the adrenalectomized young rabbit. We have, after many attempts,

failed to obtain clear results by this method, since the animals do not survive the operation for a sufficiently long time.

Two other ways of attacking the problem seemed possible, viz. by using ergotamine and iodoacetate. The former is known to inhibit adrenaline hyperglycæmia, and might conceivably act similarly on the peripheral liberation of lactate. If it did so, then it might be possible to dissociate the characteristic effects of adrenaline from those of insulin. Iodoacetate is known to prevent the post-mortem liberation of lactic acid in muscle and might, in adequate dose, prevent the liberation of lactic acid produced by adrenaline in the living animal.

*Ergotamine and the action of insulin.*

The ergotamine was used in the form of "ergotamine methan sulphate" [Sandoz] injected intravenously and the insulin was injected as usual subcutaneously. The animals were from one litter and were starved for 24 hours. The results are given in Table VII, and it will

TABLE VII. A litter of four young rabbits, starved for 24 hours.

Rabbit No.	Wt. g.	Insulin unit	Ergotamine mg.	Blood		Glycogen			Time of action
				Sugar mg./100 c.c.	Lactate mg./100 c.c.	Liver p.c.	Muscle p.c.	Total g./100 g. body wt.	
1	784	0	0	134	33	0.267	0.148	0.081	—
2	715	1.0	0	65	36	0.933	0.118	0.091	2 45
3	638	0.8	4.0	23	33	1.293	0.158	0.130	2 7
4	592	0.8	2.5	14	30	0.967	0.237	0.153	2 2

suffice to say that the effect shown in Table VI is not inhibited by ergotamine. The effectiveness of the ergotamine is shown by the much greater and more rapid fall in blood sugar in the animals injected with it. It will also be observed that the blood lactate remained the same in all the animals.

*Ergotamine and the action of adrenaline.*

The results of the combined actions of ergotamine and adrenaline are shown in Table VIII. It is sufficient to state that ergotamine, in doses large enough to inhibit adrenaline hyperglycæmia, does not prevent the redistribution of glycogen which has been demonstrated in Table III. It will also be observed that, whilst as a result of the ergotamine the adrenaline only raised the average blood sugar by about 16 p.c., the rise in lactic acid in the blood was over 80 p.c.

TABLE VIII. A litter of ten young rabbits, starved for 24 hours.

Rabbit No.	Wt. g.	Ergo- tamine mg.	Adrena- line mg.	Blood mg./100 c.c.		Glycogen		Total g./100 g. body wt.	Time of action of adrena- line
				Sugar	Lactate	Liver p.c.	Muscle p.c.		
1	625	0	0	135	23	0.754	0.430	0.204	—
2	625	0	0	115	17	0.567	0.202	0.119	—
3	525	0	0	111	16	1.164	0.193	0.130	—
4	700	0	0	137	39	1.275	0.204	0.204	—
Averages				125	24	0.940	0.292	0.164	
5	650	1.3	0.15	171	40	1.751	0.154	0.140	hr. min.
6	625	1.25	0.15	129	43	3.126	0.231	0.219	2 15
7	750	1.5	0.15	137	40	2.354	0.125	0.138	2 35
8	650	3.3	0.15	139	28	1.043	0.130	0.098	2 55
9	900	5.0	0.30	152	56	2.192	0.120	0.126	2 30
10	750	4.2	0.20	146	56	2.041	0.188	0.158	2 50
Averages				146	44	2.085	0.158	0.147	3 15

Nos. 5, 6, 7 received the adrenaline 10 min. after the ergotamine,

Nos. 8, 9, 10, 1 hour after it.

*Summary of experiments on the influence of ergotamine.*

Ergotamine does not prevent the increase in liver glycogen produced by insulin in the starving young rabbit, this increase being greater than can be accounted for by losses of glycogen from the muscles. Ergotamine, in doses sufficient to inhibit adrenaline hyperglycæmia, does not prevent the liberation of lactic acid from the muscles and the quantitative redistribution of glycogen which follows the injection of a small dose of adrenaline. It appears, therefore, that the mechanism of glycogenolysis in the liver is more rapidly paralysed by ergotamine than is that in the muscles. If, however, the dose of ergotamine is sufficient demonstrably to paralyse the peripheral vaso-constrictors in the anæsthetized animal, then the increase of lactate in the blood caused by adrenaline is also prevented (Goldblatt [1933]). Thus, with the doses of ergotamine we have used, we have been unable to dissociate the effects of insulin and adrenaline.

*Iodoacetate and the actions of insulin and adrenaline.*

The dose of the neutralized acid which could safely be given was first determined. The acid was neutralized with  $N/2$  NaOH, using methyl orange as indicator.

(a) Rabbit. 1.1 kg.

5 mg. intravenously	...	...	No symptoms
15 mg. 1 hour later	...	...	No symptoms
40 mg. 1½ hours later	...	...	Convulsions and death in 15 min.

(b) Rabbit. 1.0 kg.

10 mg. intravenously	...	...	No symptoms
15 mg. 55 min. later	...	...	No symptoms

It was decided to use doses up to 15 mg.

The results are set out in Tables IX and X and may be summarized as follows:

(1) Iodoacetate in doses up to 10 mg. does not seriously interfere with the accumulation of liver glycogen or the increase in total body glycogen which we have seen constantly to occur in these animals after insulin.

(2) With 15 mg. this effect is inhibited to a very great extent but not abolished, the total glycogen per unit body weight remaining unchanged. This amount of iodoacetate does not interfere with the rate of fall in blood sugar, nor is there any appreciable change in blood lactate.

(3) 15 mg. iodoacetate prevent the rise in liver glycogen which normally follows the injection of small doses of adrenaline but do not prevent the hyperglycæmia, the rise in lactic acid in the blood or the fall of muscle glycogen. The inability of the liver to synthesize the liberated lactic acid is reflected in the persistence of a high blood lactate. The total glycogen per unit body weight is very greatly diminished (cf. Table III).

TABLE IX. Fifteen young rabbits from two litters of approximately the same age were starved for 24 hours. Of these six were used as controls, two were injected with insulin alone, and seven with both insulin and acetoacetate as detailed in the table. Except for one animal in the last group, the litters were equally represented in all the groups.

Rabbit No.	Wt. g.	Iodo-acetate mg.	Insulin unit	Blood mg./100 c.c.		Glycogen			Time of action of insulin hours
				Sugar	Lactate	Liver p.c.	Muscle p.c.	Total g./100 g. body wt.	
1	700	0	0	77	40	0.304	0.181	0.100	—
2	700	0	0	95	39	1.057	0.189	0.130	—
3	550	0	0	100	84	0.397	0.152	0.087	—
4	700	0	0	90	68	1.949	0.267	0.199	—
5	500	0	0	67	62	0.514	0.197	0.117	—
6	600	0	0	72	63	1.096	0.226	0.150	—
Averages				83	59	0.886	0.202	0.131	
7	750	0	1	30	40	2.169	0.283	0.210	2.3
8	925	0	1	54	46	3.947	0.402	0.323	2.3
Averages				42	43	3.008	0.342	0.267	
9	800	5	1	58	59	2.587	0.250	0.216	2.3
10	850	10	1	30	89	2.156	0.212	0.179	1.3*
Averages				44	74	2.372	0.231	0.198	
11	650	15	1	30	31	1.526	0.258	0.178	2.3
12	750	15	1	48	68	0.809	0.160	0.108	2.3
13	725	15	1	62	48	1.135	0.136	0.109	2.3
14	700	15	1	48	46	0.875	0.173	0.116	2.3
15	700	15	1	45	74	1.469	0.138	0.130	2.3
Averages				48	53	1.163	0.173	0.128	

\* Convulsion.

TABLE X. Eight young rabbits from the same litter were starved for 24 hours. Three were used as controls and five were injected with iodoacetate and adrenaline.

Rabbit No.	Wt. g.	Iodoacetate mg.	Adrenaline mg.	Blood mg./100 c.c.		Liver p.c.	Glycogen		Time of action of adrenaline hours
				Sugar	Lactate		Muscle p.c.	Total g./100 g. body wt.	
1	650	0	0	137	56	Lost	0.400	—	—
2	650	0	0	118	28	2.139	0.574	0.358	—
3	650	0	0	113	26	1.065	0.428	0.255	—
Averages				123	37	1.602	0.467	0.307	—
4	600	15	0.15	229	51	1.098	0.194	0.136	2.3
5	600	15	0.15	236	39	1.055	0.245	0.161	2.3
6	750	15	0.15	168	64	2.457	0.239	0.226	2.3
7	600	15	0.15	187	46	1.205	0.258	0.181	2.3
8	625	15	0.15	227	85	1.164	0.232	0.162	2.3
Averages				209	57	1.396	0.234	0.173	—

It is clear that the increase of lactate in the blood caused by adrenaline is not affected by iodoacetate in doses of 15 mg. Hence, were the accumulation of liver glycogen shown in Rabbits Nos. 7 and 8 in Table IX due to resynthesis from lactate released from the muscles, we should have obtained a great rise in blood lactate in Rabbits Nos. 11 to 15, in which the formation of new glycogen in the liver was practically abolished. (Rabbit No. 10, Table IX, illustrated that the peripheral release of lactate was easily possible.) We consider that this failure of the lactate to be increased constitutes evidence against the adrenaline hypothesis. In Table IX the last group of animals, in spite of the fall in blood sugar, showed no change in total body glycogen, whereas in the litter of Table X the total fell by over 40 p.c., no appreciable loss having occurred by the urine. Iodoacetate, whilst not giving the unequivocal evidence sought for, served to emphasize the quantitative differences between the effects of insulin and adrenaline in these animals.

#### DISCUSSION.

The most recent contribution to the problem presented by these young animals is that of Corkill [1932], in which it is shown that diphtheria toxin, in doses that were too small to abolish the power of the liver to make glycogen from glucose, completely prevented the deposition of liver glycogen which ordinarily follows the injection of insulin or adrenaline. The toxæmia is accompanied by a distinct resistance to insulin as far as the blood sugar is concerned, but this resistance can be overcome by ergotoxine, which supports the view that adrenaline secretion is



increased during the toxæmia. But ergotoxine does not allow of a deposition of liver glycogen by the action of insulin in the toxæmic young rabbit. Corkill has thus also reached an impasse in the elucidation of the source of the new glycogen. In regard to these very interesting experiments it would be important to determine whether in toxæmia there occurred a large rise in blood lactate, and whether injected lactate gave rise to liver glycogen. By intravenous injection of *d*- or *i*-lactate it is easy to produce a deposition of liver glycogen in normal young rabbits, and it may be that the toxæmic liver may have lost the power to form glycogen from lactate whilst retaining that from glucose. Should this prove to be the case, the necessary link in connecting the anomalous action of insulin with the adrenals might become clearer. The fact that, as we have seen, ergotamine permits both the insulin and adrenaline effects to take place and that iodoacetate and diphtheria toxin [Corkill] prevent them, undoubtedly gives qualitative support to the hypothesis that there is some link between the two; but on quantitative grounds we consider that there are outstanding differences between the observed changes and what might reasonably be expected if the effects following the injection of insulin in these animals were really due to adrenaline. Further facts must be obtained before a definite decision can be reached.

#### CONCLUSIONS.

1. Insulin and adrenaline under properly controlled conditions produce a very marked increase in liver glycogen in the starving young rabbit.
2. In the case of insulin this increase may or may not be associated with a fall in muscle glycogen. The effect on the total glycogen of the body is most frequently an increase.
3. In the case of adrenaline in non-glycosuric doses, the increase in liver glycogen is always associated with a fall in muscle glycogen. With a properly adjusted dose there is a quantitative redistribution of glycogen between the muscles and the liver, the total remaining unchanged. With larger doses there is a loss in both situations.
4. Ergotamine, in doses up to 5 mg. intravenously, does not prevent the action of insulin or of adrenaline on the distribution of glycogen.
5. Iodoacetate, in doses of 15 mg., prevents the accumulation of liver glycogen usually produced by insulin and adrenaline in young rabbits. The final result in the case of adrenaline is a loss of glycogen from both liver and muscles; in the case of insulin the total glycogen was not changed.

6. The action of insulin in these animals is not accompanied by such an increase in blood lactate as would justify the belief that the adrenal secretion is the cause of the increase in liver glycogen which always follows such action.

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THE NORMAL BEHAVIOUR OF THE ISOLATED  
UTERUS OF THE GUINEA-PIG, AND ITS  
REACTIONS TO ŒSTRIN AND OXYTOCIN.

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THE object of this paper is to amplify the substance of two communications by Marrian and Newton [1932, 1933], on the action of œstrin and oxytocin on the uterine contractions of guinea-pigs. As a rule this animal has been adhered to, but mice were employed for a few experiments. The animals have been used in four conditions, viz. immature virgin, mature non-pregnant, and oöphorectomized, and it is proposed to arrange the findings under these four headings.

The method used consisted of suspending the uterus, or a strip of it, in a bath of oxygenated saline solution, at 37° C., and recording its contractions by means of a light lever and frontal writing point. Except where otherwise stated, the animal was killed by a blow on the head, the blood drained from it, and the uterus removed immediately. The solution and apparatus used were those suggested by Burn and Dale [1922].

THE IMMATURE VIRGIN UTERUS.

*Normal behaviour.* The uterine horns of female guinea-pigs of about 200 g. weight, and reared apart from males, are generally regarded as satisfactory for the assay of the posterior pituitary oxytocic principle. This is partly because, when suspended in the muscle bath, they maintain a constant degree of relaxation, their spontaneous contractions being insignificant and irregular. The amplitude of these contractions may be reduced, up to a point, by applying a load to the lever. Whatever be this load, however, after about 2 hours the spontaneous activity of the uterus increases, and the base line of the tracing rises, i.e. a certain amount of tonic contraction takes place (Fig. 1).

*Action of oxytocin.* This is well known, and requires only a brief description. On adding oxytocin to the bath, a strong contraction of the muscle takes place, and for a few minutes a state of steady tonus is maintained. The level of this is constant for the same submaximal dose of oxytocin; if the latter is increased or decreased, the level is respectively higher or lower. This property of the uterus is the basis for the assay of pituitrin, and is fully discussed by Dale and Laidlaw [1912], and Burn and Dale [1922]. If the dose of oxytocin is left in the bath, the tonus falls

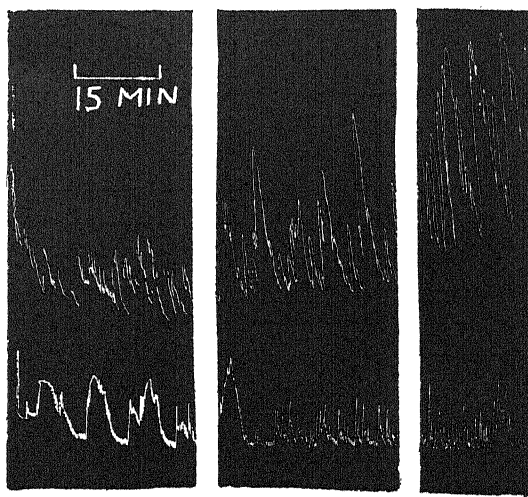


Fig. 1. Virgin guinea-pig, 220 g. Upper and lower tracings taken simultaneously from left and right uterine horns respectively. The solution bathing the right horn was saturated with ketohydroxyoestrin. First space=interval of 1 hour; second space=interval of  $\frac{1}{2}$  hour.

after a time, and the tracing is broken by fairly large, irregular, spontaneous contractions. If the solution is changed to a fresh one immediately the contraction has attained its initial steady value, the relaxation is rapid, and the spontaneous contractions no greater than before. After some hours the preparation becomes too sensitive for quantitative work, responding with a maximal contraction to different and formerly sub-maximal doses of oxytocin.

*Action of oestrin.* (a) Effect on normal behaviour. One horn of the uterus was suspended in the plain saline solution, and the other in a similar solution saturated with pure crystalline ketohydroxyoestrin. There was no significant difference between the spontaneous contractions in the

two cases, and the only difference appeared to be that the increase in tonus and general activity, which normally comes on after a few hours, was delayed in the horn exposed to the cestrin solution (Fig. 1). The

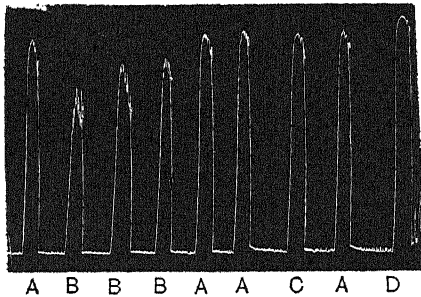


Fig. 2.

Fig. 2. Virgin guinea-pig, 210 g. Contractions produced by addition to bath (60 c.c.) of the following: *A*=0.016 unit oxytocin+0.1 c.c. absolute alcohol. *B*=0.016 unit oxytocin+0.2 mg. ketohydroxyœstrin in 0.1 c.c. alcohol. *C*=0.032 unit oxytocin+0.2 mg. ketohydroxyœstrin in 0.1 c.c. alcohol. *D*=0.032 unit oxytocin+0.1 c.c. absolute alcohol. Interval between doses 15 min.; bath washed out after each dose, and drum stopped during relaxation of muscle. The last four contractions show that at least an additional 0.016 unit oxytocin is required to overcome the effect of 0.2 mg. of ketohydroxyœstrin.

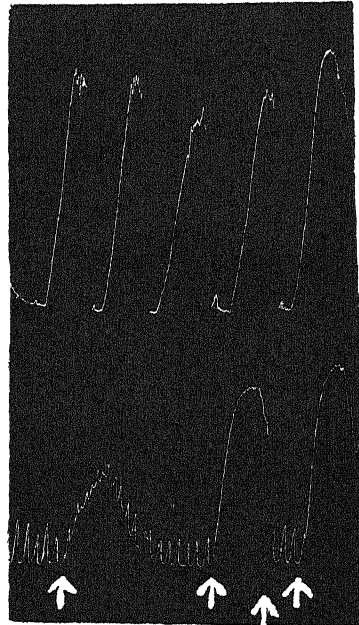


Fig. 3.

Fig. 3. Upper tracing. Virgin guinea-pig 240 g. Contractions: 1, 2, and 5 produced by 0.02 unit oxytocin+0.1 c.c. absolute alcohol; 3 and 4 by 0.02 unit oxytocin+0.2 mg. triacetate of trihydroxyœstrin in 0.1 c.c. alcohol.

Lower tracing. Virgin guinea-pig, 200 g. Arrows: 0.1 mg. monomethyl ether of ketohydroxyœstrin in 0.5 c.c. alcohol; 0.04 unit oxytocin; fresh bath, and interval of 30 min.; 0.04 unit oxytocin+0.05 c.c. absolute alcohol.

reason for this late activity is in any case obscure, and it is doubtful if it is permissible to draw conclusions from the later part of experiments such as these.

(b) Effect on reaction to oxytocin. The oxytocin used in these experiments was a partially purified solution prepared by J. M. Gulland ("Kamm solution," Gulland and Newton [1932]). It was diluted, and the amount found which would produce a contraction of suitable size when added to the muscle bath (capacity on this occasion 100 c.c.). It was then ascertained that the simultaneous addition of 0.1 c.c. absolute alcohol was without effect on the response to this dose. Finally a test was performed in exactly the same way as if two different solutions of oxytocin were being compared, except that the dose of oxytocin was constant, and the difference lay in the simultaneous addition of 0.1 c.c. of alcohol in one case, and 0.2 mg. œstrin dissolved in 0.1 c.c. of alcohol in the other. The result of this experiment has previously been reported, namely, that œstrin markedly reduces the response to oxytocin [Marrian and Newton, 1932]. The accompanying tracing (Fig. 2) gives a rough idea of the magnitude of this reduction.

*The action of derivatives of œstrin.* (a) The triacetate of trihydroxy-œstrin. The effect of this is the same as that of ketohydroxyœstrin (Fig. 3).

(b) The monomethyl ether of ketohydroxyœstrin. This itself produces a symmetrical contraction and relaxation of the muscle. In investigating its effect on the response to oxytocin, this necessitates some irregularity in the timing of the doses, but so far as these experiments warrant any conclusion, it is that the response to a dose of oxytocin, after the contraction from the methyl ether has subsided, is depressed (Fig. 3).

*Effect of pregnandiol.* The contractions from oxytocin are slightly diminished in the presence of pregnandiol, but when this has been removed, the original degree of contraction with oxytocin cannot be reproduced. It is therefore impossible to say whether this is a permanent, slight effect of pregnandiol, or whether the sensitivity of the uterus is gradually diminishing, and the pregnandiol is inactive. The same result was obtained, however, on several occasions. In any case, the action of pregnandiol is not comparable with that of œstrin.

#### *Discussion.*

These experiments throw little light upon the virgin uterus as an organ; the ultimate effect of oxytocin is simply to increase its muscular activity, but this is as irregular as before. Œstrin has at the most a questionable depressor influence. Regarded as a means for measuring the activity of oxytocin, however, the uterus assumes an apparent importance, for it is clear that this activity is impaired *in vitro* by the presence

of oestrin, and certain of its derivatives. In studying the interaction between the two hormones, it cannot be too strongly emphasized that results may vary according to the technique adopted and the animal used. The sexual development and condition of the animal may also have an influence. The only experiments bearing on the present work are those previously performed by the same technique. Heller and Holtz [1932] and Jeffcoate [1932] stated that oestrin was without effect on the response to oxytocin of the virgin uterus *in vitro*, and although they confirmed their results with crystalline oestrin, it is possible that they had difficulty in getting a sufficient concentration of this substance into solution. Burn and Bourne reported [1928] that oestrin increased the sensitivity of the uterus to oxytocin, but withdrew their experiments [1932] when crystalline oestrin became available.

The fact that it is now clearly demonstrable that oestrin impairs the action of oxytocin, while it is not so clear that it interferes with the normal behaviour of the uterus, suggests immediately that oestrin may have no action on the muscle *in vitro*, but may throw out of action, by physical or chemical means, a part of the oxytocin. To set against this is the fact that the activity of oxytocin can be accurately measured, whereas it is difficult to interpret what is and what is not variation in the extremely irregular normal contractions, so that a slight effect on the latter may be overlooked.

#### THE UTERUS OF THE OÖPHORECTOMIZED ANIMAL.

*Normal behaviour.* The uterus undergoes involution and coincidentally the spontaneous activity dies away, so that 3 months after oöphorectomy a preparation is obtained which is quiescent in the muscle bath. In behaviour it resembles the virgin uterus, except that the spontaneous contractions are smaller and more sluggish; if left in the bath for some time, it may show slow variations in tonus, but does not become active like the virgin organ. Any deviation from this condition is very likely to be due to regeneration of ovarian tissue [Parkes, Fielding and Brambell, 1927], and to guard against this about  $\frac{1}{4}$  in. of each uterine horn has been regularly removed with the ovaries.

*Action of oxytocin.* Addition of a suitable amount of oxytocin to the bath causes a slow contraction of the muscle, which becomes steady at a level corresponding to the dose of oxytocin given. On one or two occasions when the latter was left in the bath for a long time, the contraction of the muscle appeared to be sustained indefinitely. The height of the contraction may not be attained until as long as 8 or 10 min., and after

hour or two, all types of tracing tended to settle down to the second type. In comparative experiments, therefore, it is probably only legitimate to use the first hour and a half of the tracing.

The effect of increasing the temperature is to increase the frequency of the contractions (Fig. 6); their amplitude varies with the length of the strip, and therefore with the size of the uterus if the whole horn is taken. For these reasons more stress is laid upon the form of the contractions than upon their frequency and amplitude. The two horns from the same

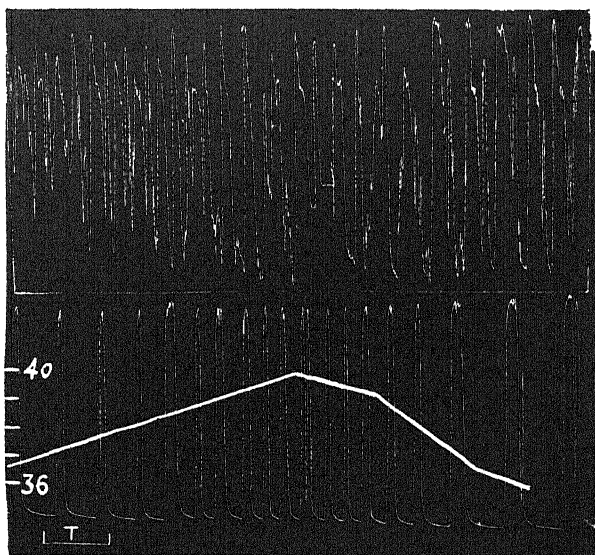


Fig. 6. Typical tracings from the uteri of two adult guinea-pigs, 550 and 520 g. respectively. The lower tracing shows the effect of temperature change upon the contractions (scale and continuous line).  $T=15$  min.

uterus give as a rule the same type of contraction, although one or two exceptions to this have been encountered; no error from this source is likely to occur if sufficient experiments are done.

*Effect of œstrin.* (a) Some of the saline solution was saturated with pure crystalline ketohydroxyœstrin (probably between 0.2 and 0.4 mg./100 c.c.). One horn of the uterus was then put up in this solution, and the other in the original saline solution. There was not sufficient difference between the form of contraction in the two cases to warrant any statement as to the action of œstrin given in this manner.

(b) Crystalline ketohydroxyœstrin was dissolved in absolute alcohol, 2 mg./100 c.c., and each horn of the uterus suspended in the ordinary way



in the saline solution. After the tracing had become established 0.1 c.c. of the œstrin solution was added to one bath and 0.1 c.c. of absolute alcohol to the other. In one experiment this procedure was repeated twice, so that finally three times the usual quantities of œstrin solution and alcohol respectively were present. In neither horn was the tracing appreciably altered by this treatment, and this was the rule in these experiments.

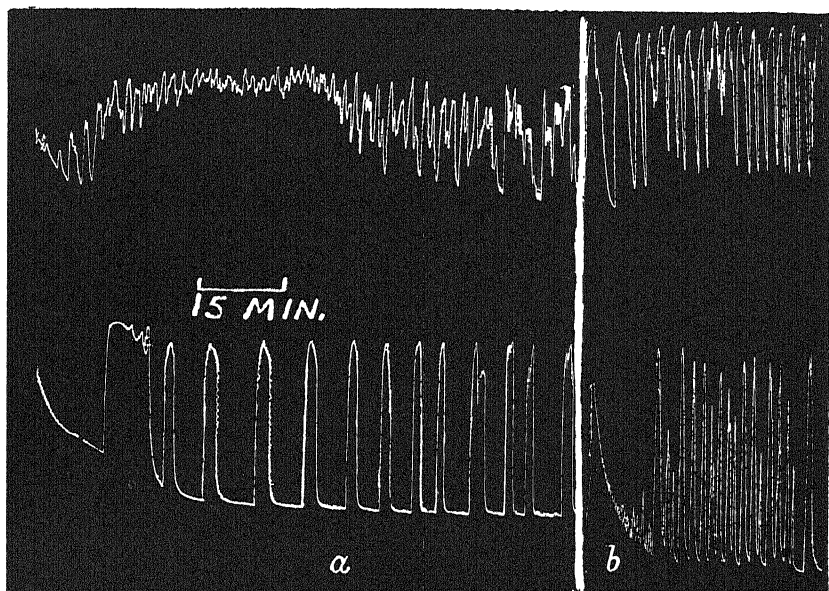


Fig. 7. (a) Tracings from the two horns of an adult guinea-pig before (above) and after (below) œstrin injection respectively (see text). (b) Corresponding tracings from the two horns of the least satisfactory control guinea-pig, in which the second tracing most nearly resembled the tracings after œstrin injection. (The animal was on œstrus when the upper record was taken.)

(c) Two guinea-pigs of approximately equal age and weight (300–350 g.) were anaesthetized with ether, one uterine horn removed aseptically from each, and tracings from these horns taken in the usual way. The day after the operation a course of subcutaneous injections was begun; one animal receiving œstrin and the other the solvent (10 p.c. alcohol). The injections were given hourly (except through the night), and continued for 48 hours; this represented about 25 c.c. of fluid, and 0.6 mg. of œstrin. 18–24 hours after the last dose the animals were killed, the

remaining horns removed, and their contractions recorded. The types of contraction obtained from the horns of the control guinea-pig agreed fairly well, in spite of the interval between the records. In the other animal, however, the effect of the œstrin was distinctly seen (Fig. 7). The contractions recorded from the second horn were relatively infrequent, regular, and apparently maximal; the muscle was fully relaxed between them, and this, together with the absence of minor contractions, gave a very definite base line to the tracing. This result was invariable.

### *Discussion.*

It is clear that the injection of œstrin does modify the *in vitro* contractions of the uterus, and it would be expected that the same effect would be apparent when œstrin is produced by the animal itself, *i.e.* during œstrus. One has not been able to demonstrate this for the guinea-pig, possibly because of the age and uncertain history of the animals used. It has been stated, however [Frank, Bonham and Gustavson, 1925; Durrant and Rosenfeld, 1931; Harne, 1931], that in the rat the type of activity exhibited by the excised uterus during œstrus is similar to that just described after injections of œstrin into the guinea-pig. Reynolds [1931], recording from a fistula, has found that the uterus of the rabbit is inactive during dioestrus and after oöphorectomy, but active during œstrus and after injection of œstrin. This agrees with the present findings in oöphorectomized guinea-pigs (although these require confirmation, as previously pointed out), but it would be ambiguous to say whether normal guinea-pig uteri are more or less active after œstrin injection; the activity seems rather to change qualitatively and become more coordinated. Azuma and Soskin [1932], using a fistula, found uterine motility to be diminished in the dog 24 hours after œstrin injection.

### THE UTERUS OF THE PREGNANT ANIMAL.

*Normal behaviour.* In the pregnant guinea-pig only one horn of the uterus is usually involved; this is referred to as the "pregnant horn," and the other as the "non-pregnant horn." The former is enormously hypertrophied, and its wall is so thin as to be transparent. In the majority of the experiments only one foetus has been found: this attains at term a length of about 11 cm. measured from the frontal bone to the posterior tip of the spinal column. In the absence of any knowledge of the previous history of the animals, the length of the foetus has been used as a guide to

the stage of pregnancy. On several occasions two foetuses have been found, but the behaviour of the uterus on these occasions has been similar to that observed when the foetus has been a single one of the same length as each of the pair. The non-pregnant horn is larger than that of a non-pregnant animal, and is occluded at the lower end by a plug of mucus.

Longitudinal strips cut from the pregnant horn give in the early stage of pregnancy a series of spontaneous contractions which are fairly regular and maximal, with a well-marked base line between. Later in pregnancy

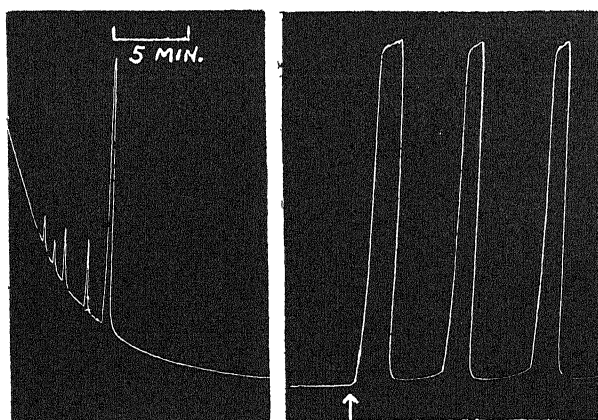


Fig. 8. Pregnant guinea-pig, foetus (one) 7 cm. Longitudinal strip from pregnant horn, showing rapid relaxation in bath. The gap in the tracing represents 50 min., and during this time no spontaneous contractions took place. At the arrow, 0.01 unit oxytocin was added to the bath (100 c.c.).

these contractions become less frequent as the record progresses, and may cease altogether. Later still in pregnancy the strip rapidly relaxes on being placed in the bath, and gives no spontaneous contractions whatever; the tracing appears like a line drawn with a ruler (Fig. 8). It is not yet clear whether spontaneity returns at term; the whole series of experiments is being repeated with animals whose stage of pregnancy is accurately known. Throughout pregnancy the non-pregnant horn gives maximal, fairly regular spontaneous contractions.

*Action of oxytocin.* This has been investigated only on the inactive strips of the uterus of late pregnancy (foetus 9-10 cm.). When oxytocin is added to the bath in which these are suspended, they break into perfectly regular, smooth, maximal contractions (Figs. 8 and 11), which apparently

continue so long as the oxytocin is left in the bath. The concentration of oxytocin necessary to bring these about is of the same order as that usually required to produce a nearly maximal contraction of the virgin uterus, *i.e.* 0.01–0.02 unit in 100 c.c. The exact threshold dose for an individual strip depends in part upon the load carried by the writing lever, *i.e.* upon the tension of the muscle, as the following experiment shows. If a very small dose of oxytocin be added to the bath the tracing continues unaltered as a straight line: if the dose be increased a point is reached at which the base line begins to rise slowly. This rise continues for 2 or 3 min., when suddenly a maximal contraction occurs, with the usual almost instantaneous rise of the lever; after the contraction the

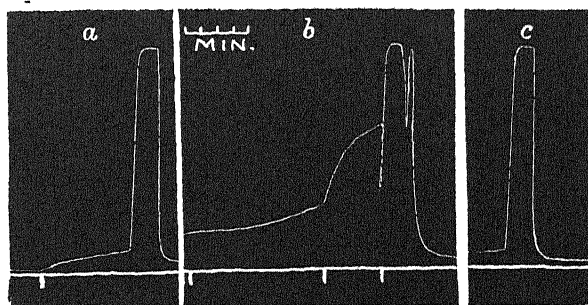


Fig. 9. Pregnant guinea-pig. Length of foetus (one) 9 cm. No spontaneous contractions. (a) Effect of 0.015 unit oxytocin. (b) Later, after removal of load from lever so that muscle was just balanced. Signals: (1) 0.02 unit oxytocin; (2) same; (3) load applied to lever. (c) Contraction 15 min. later; similar contractions followed regularly at 15 min. intervals.

lever returns to the original base line, and there is a relatively long interval before the next contraction, which is also preceded by the slow shortening. A further increase in the amount of oxytocin present yields the usual series of contractions, which are more frequent and without, or almost without, the preliminary shortening. If now the load on the lever be removed, so that its weight is just sufficient to balance the strip, it is found that what was before an adequate dose of oxytocin, now gives rise only to an exaggerated version of the slow type of contraction, and although this may produce considerable shortening of the strip, there may be no sign of the usual brisk maximal contraction and relaxation. Application of a load to the lever, however, immediately initiates such a contraction, and further uncomplicated contractions occur at regular intervals (Fig. 9).

The importance of tension is shown also by Fig. 10, which shows that the frequency of contraction varies directly as the load on the lever. The rhythm also varies with the temperature of the bath.

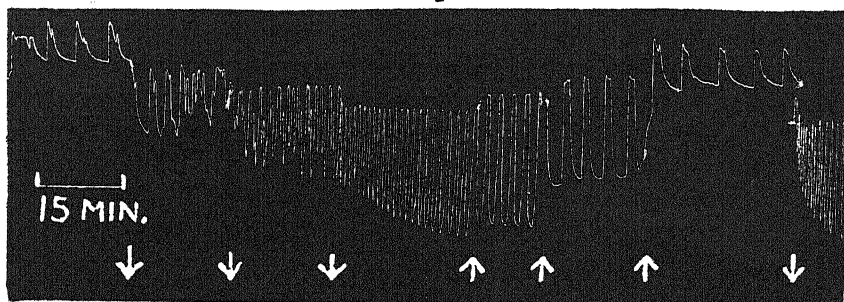


Fig. 10. Pregnant guinea-pig; length of foetus (one) 10 cm. Longitudinal strip from pregnant horn. Tracing after 0.03 unit oxytocin, with lever just balanced. Arrows indicate application or removal of load, as follows: (1) +0.33 g.; (2) +0.48 g.; (3) +0.60 g.; (4) -0.60 g.; (5) -0.48 g.; (6) -0.33 gm.; (7) +1.41 g.

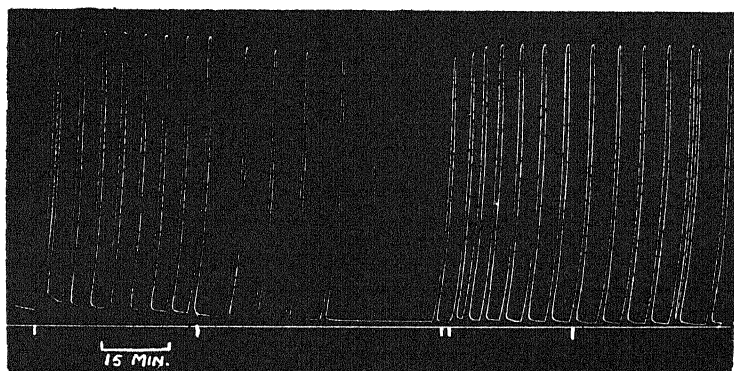


Fig. 11. Pregnant guinea-pig. Length of foetuses (two) 9 cm. Strip of pregnant horn in 100 c.c. bath. At signals: (1) 0.025 unit oxytocin; (2) 0.2 mg. ketohydroxyoestrin in 1 c.c.  $N/10$  NaOH + exactly equivalent amount of HCl; (3) fresh solution in bath; (4) 0.025 unit oxytocin; (5) 1 c.c.  $N/10$  NaOH + exactly equivalent amount of HCl.

An excessive dose of oxytocin tends not only to initiate contractions, but also raises the base line between these, so that the uterus is in a state of continued partial contraction.

*Action of oestrin.* If a large amount of oxytocin has been used, so that rhythmic contractions are superimposed on a permanent tonus, the addition of oestrin to the bath reduces this tonus, and full relaxation

occurs between the beats. In such circumstances the rhythm may be accelerated [Marrian and Newton, 1932]; what happens when still more œstrin is added cannot be determined, owing to the difficulty of getting large amounts into solution. If just sufficient oxytocin to give a series of rhythmic contractions, with no general rise of base line, is present, then, on treatment with œstrin, the contractions are diminished in frequency and amplitude. In other words, the effect of œstrin is to neutralize that of oxytocin. These effects have been obtained using alcoholic and alkaline solutions of œstrin, and the action of the solvent has been controlled (Fig. 11).

#### GENERAL DISCUSSION.

There seems to be no doubt that the muscular activity of the uterus may be modified by injecting preparations of œstrin into animals. Some of the work already quoted suggests that œstrin stimulates, and some that it depresses the uterus, but results from the same animal appear consistent. There is probably, therefore, a species difference, but this cannot with confidence be stated until it is established that the uterus as studied by fistula in the intact animal and the excised uterus behave in the same way at the same time.

The fact that the effect of œstrin injection can so easily be traced in the activity of the excised uterus points to some stable alteration having taken place in the muscle. The most obvious change of this sort is an enlargement of the uterus, and this may be associated with increase in size, number, or both, of the muscle fibres. Andrei [1924] has reported hyperplasia in the œstrous rabbit, and Anopolsky [1928] increase in length of the tubal muscle fibres of the sow during heat. Before attributing a "pharmacodynamic" action to œstrin [Brouha and Simonet, 1927], it is important to decide whether such structural alterations in the muscle would be sufficient to account for a qualitative change in activity such as is seen in the guinea-pig. At first sight the correlation between activity and size, as seen in virgin, mature and involuted uteri, seems straightforwardly quantitative; but the large uteri of large guinea-pigs frequently yield tracings suggestive of those obtained after œstrin injection, and the non-pregnant horn of the pregnant animal, which is considerably enlarged, certainly gives this kind of tracing. Further, if the characteristics of this type of activity (diminished frequency of contraction, the maximal nature of such contractions as do occur, and the tendency to complete relaxation between the beats) were exaggerated and pushed to the limit, the same stages would be anticipated as do actually

occur with progressive hypertrophy in the pregnant horn of the pregnant animal. The dissimilarity between the activity of the pregnant and non-pregnant horns (apparently overlooked by Knaus [1927], who used the latter as a criterion for the former) emphasizes the importance of structural or mechanical conditions as distinct from hormonal factors. There is at present insufficient evidence to decide whether the change in size of the uterus after oestrin injection has anything to do with the coincident change in its activity, but this possibility cannot be ignored.

In the experiments where oestrin is added to the muscle bath, instead of being injected previously into the animal, the most striking phenomenon is its antagonism to oxytocin, but it is not legitimate to assume that this is physiological. There seems to be no conclusive evidence as to the normal interaction between these two hormones. Parkes [1930] produced immediate abortion in a certain percentage of mice by following up 12 hours of oestrin injection with the injection of 1 unit of pitocin, to which the mice were normally insensitive. He argued from this that there was some immediate action on the muscle, sensitizing it to pitocin; this action may not have been due to oestrin, however, since no crystalline preparation was available. Moreover, Illingworth, Marshall and Robson [1932] and Jeffcoate [1932] claim that there is a substance sensitizing the uterus to pitocin in extracts of urine free from the known sex hormones. Brouha and Simmonet [1927] describe the types of contraction produced in the isolated uteri of guinea-pigs by pituitrin, when these are normal when killed, or previously treated with follicular extracts. After oestrin, instead of a tonic contraction, a series of rhythmic contractions are obtained; this agrees with our findings, but does not throw light on the sensitivity of the uterus. Pompen [1933], however, taking records from the living animal by means of Trendelenburg's method [1913], definitely states that oestrin injection lowers the threshold to pituitrin in rabbits, cats, and guinea-pigs.

#### SUMMARY AND CONCLUSIONS.

1. The types of uterine contraction met with in the isolated uteri of guinea-pigs, immature, mature, pregnant and oöphorectomized, are described, together with certain temperature and tension effects.

2. Oestrin, *in vitro*, has no certain action upon the immature or mature uterus, but oestrin previously injected into the intact adult animal profoundly modifies the activity of the uterus subsequently excised. The possible relation of this change in activity to hypertrophy of the muscle is discussed.

3. The action of oxytocin on the immature, the pregnant, and involuted uterus is described, and it is shown that this action is in each case partially neutralized by the simultaneous presence of œstrin in the muscle bath. This effect of œstrin is not necessarily related to its true physiological action.

4. The uteri of guinea-pigs 3-4 months after oöphorectomy can be used for the assay of the oxytocic pituitary principle, but the method is tedious and probably only of value for confirmatory purposes.

I wish to thank Dr G. F. Marrian, who, by providing crystalline œstrin and by much valuable assistance, has made this work possible.

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## THE EFFECT OF THE CARDIAC CONTRACTION UPON THE CORONARY FLOW.

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IN spite of the extensive researches made during recent years, some of the most fundamental questions concerning the coronary circulation still remain a field of considerable debate. The question whether the blood supply to the heart muscle is maximal during the period of systole or during the period of diastole seems to be the centre of most violent and uncompromising exchanges of opinion.

A comprehensive review of the literature on the coronary circulation was recently made by Condorelli [1932]. Amongst other problems, the author deals in a purely objective and unbiased way with the different opinions about the extent to which the cardiac contraction may affect the coronary flow. Condorelli concludes that the blood supply to the left ventricle during the period of systole is but little affected by the systolic rise of the aortic blood-pressure. A second monograph on the same subject by Hochrein [1932] is chiefly devoted to a restatement of the author's own opinions and to an attempt to prove that the maximal blood supply to the heart occurs, as in any other organ, during the period of systole. This conclusion is supported by references to the author's former publications. No new facts are provided, and the criticism of his experiments which was made in communications from this laboratory [Anrep, Davis and Volhard, 1931; Davis, Littler and Volhard, 1931] is left unanswered although it is mentioned. We refrain from any polemical discussion of this problem, as we prefer to subject the question to a still further experimental test.

If the coronary artery were suddenly and completely blocked for a short time during some definite period of the cardiac cycle, the effect of this block on the blood supply to the heart muscle would depend on whether the clamping period coincided with the period of maximal or of

minimal blood flow through the artery. If the blood flow through the coronary artery were entirely uniform throughout the cardiac cycle, repeated short periods of clamping would always have the same effect upon the blood flow, and it would be immaterial whether these periods of clamping fell within the systole or the diastole. So long as the blood flow through the coronary artery is not uniform, however, and so long as periods of larger and smaller flows stand in some definite relation to the cardiac cycle, clamping of the coronary artery which coincided with the periods of reduced flow would produce a small diminution of the flow through the artery, while clamping which coincided with periods of large flow would produce a large diminution. If at some definite phase of the cardiac cycle the flow is at a standstill, clamping of the artery which exactly coincided with this phase should produce no change in the blood flow whatever. By gradually shifting the period of clamping so as to make it fall within different stages of the cardiac cycle, it should be possible to determine at which stage the clamping produces the minimal effect and thus to decide whether the blood supply to the heart is smaller during systole or during diastole.

If a glass tube is provided at each end with a rotating tap, the periods of closing and opening of which could be made to coincide or to alternate, the effect of closing these taps upon the flow of liquid through the tube would be maximal in the case of alternate closing; and if the closure of one tap were to coincide exactly with the opening of the other, the flow would be reduced to zero. Approximately similar conditions should hold for the heart, except that in this case we deal not with a rigid tube but with blood vessels which have certain elastic properties. In this respect one could compare the effect with that produced by turning two taps which are placed at the ends of a rubber tube. In the case of alternate closure of the taps, the rubber tube would be distended when the distal tap was closed and the proximal tap opened. When the proximal tap was closed and the distal one was opened, the rubber tube would empty itself to an extent determined by its elasticity. Therefore the flow of liquid would be considerably reduced but not completely stopped.

A constant effect of periodical clamping of an artery can only be hoped for if the clamping always takes place during some definite period of the cardiac cycle, and if the length of each clamping period is maintained constant. The most definite results should be expected if the length of clamping is made equal to the length of the period of the minimal or of the maximal blood flow through the artery. The apparatus necessary for this purpose should therefore be constructed so as to allow the clamping

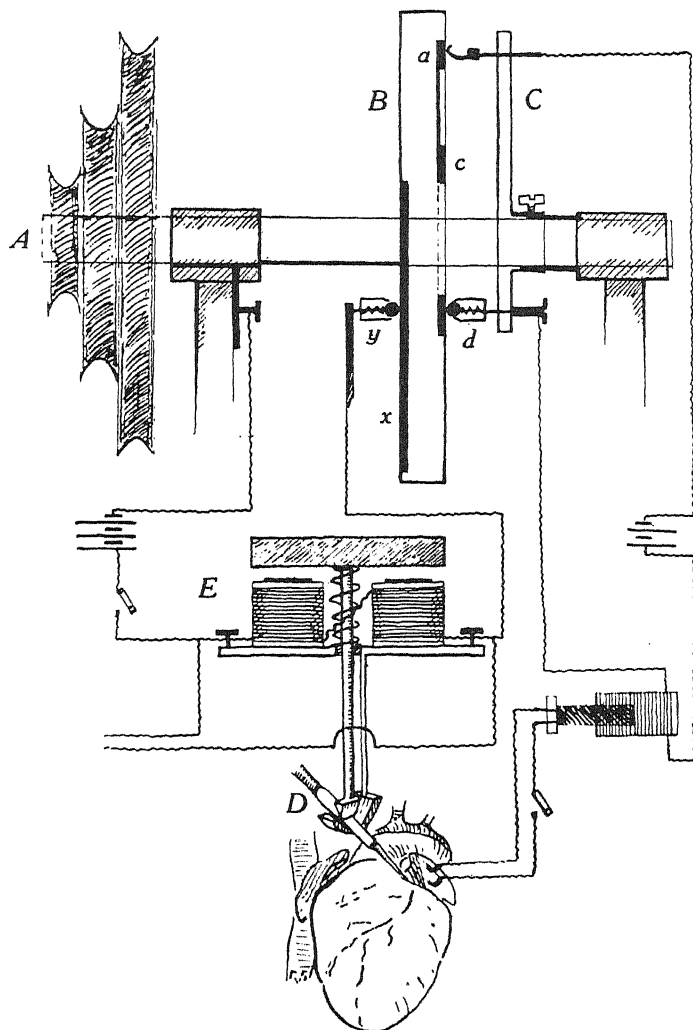


Fig. 1. The clamping apparatus. *A* are pulleys for driving the apparatus. *B* is an ebonite disk carrying a spring contact *a* for the "driving circuit" and a brass plate contact *x* for the "clamping circuit." The arm *C* is stationary and is insulated from the shaft; it can be fixed in any position in relation to the rotating disk, so that the moment at which the single induction shocks affect the heart can be changed; *c* is a brass circle which is in constant contact with the spring ball *d*. The brass plate *x* forms a sector of  $180^\circ$  of the disk close to the shaft and  $36^\circ$  at the periphery; the spring ball contact *y* is fixed on a swing arm which can be adjusted in any desired position so as to activate the clamping circuit for any duration between 0.1 and 0.5 of a revolution of the disk. *E* is the electromagnet and *D* the plunger-like extension of its armature which clamps a short piece of rubber tubing connected with the coronary cannula.

to be made at any desired phase of the cardiac cycle and to be continued for any desired length of time. We are greatly indebted to the instrument maker of the University, Mr F. W. King, for the construction of an apparatus which fulfilled these requirements (see Fig. 1). The important feature of the apparatus is that it can activate two independent electric circuits in such a way that the moment of activation of either of them and the duration of the activation of one can be altered at will. One circuit is connected with a pair of electrodes placed on the auricle or ventricle of the heart. Through these electrodes, by means of single induction shocks, the heart is driven to beat at a constant rate between 100 and 160 beats per minute. The contact for this circuit is made between a wire fixed on the rotating ebonite disk of the apparatus and a stationary spring contact. The second circuit is used to activate an electromagnet, by means of which the actual blocking of the coronary blood flow is effected. One of the contacts for this circuit is made out of a brass plate of a special shape and is fixed to the same rotating disk. The second contact for this circuit can be placed in such a position relative to the rotating disk that it can be made to slide over the brass plate contact for any desired fraction of the disk, cutting through sectors from 36 to 180°. On setting the disk in rotation, the clamping circuit can thus be activated for any length of time between one-tenth and one-half of the period of rotation. The position of the driving contact can be shifted round the whole disk, and thus the beginning of the activation of the clamping circuit can be made to coincide with the activation of the driving circuit, or can be made to follow it at any time within the period of revolution of the disk. In this manner the clamping of the coronary artery can be made to occur during any phase of the cardiac cycle and to continue from 0.1 to 0.5 of the cycle length.

The clamping was made by an electromagnet, the armature of which was connected with a plunger which could slide into a little groove. The electromagnet was strong enough to lift a weight of 2 kg. and to clamp a thin-walled rubber tube connected with a water pressure of 500 mm. Hg. The excursions of the electromagnet did not exceed 4 mm. and the clamping was extremely rapid. The registration of the clamping was made by means of a small electromagnetic signal connected with the clamping circuit. In order to determine the rapidity of the clamping and the accuracy of its registration by the signal, the following tests were made. A reservoir containing water was placed at a suitable height and connected by means of a metal tube to a cannula, the nozzle of which was provided with a short piece of thin rubber tubing. This rubber tube was

placed in the groove under the plunger of the electromagnet, and the flow of water through the tube was measured before and during periods of clamping. The disk bearing the contact was set in rotation at some definite rate, and the length of each individual clamping of the tube was changed between the measurements of the flow by one-tenth of a revolution of the apparatus. Even under the best conditions one would hardly expect that, with each prolongation of the clamping time by one-tenth, the flow would diminish by exactly 10 p.c. The apparatus, however, worked quite satisfactorily, as can be seen from the following example. The free flow of water through the tube was 55 c.c. per min. The clamping contact was set in rotation at 150 revolutions per min., and the length of each clamping was increased by one-tenth of a revolution from 0.1 to 0.5, an increase of 0.04 sec. each time. The corresponding diminutions of the flow with each increase of the length of clamping were 9.0, 19.0, 29.0, 39.5 and 49.0 p.c.

Hot-wire registration of the outflow of water from the reservoir, or of the outflow from the cannula beyond the place of clamping, showed that the clamping was extremely rapid, the flow being arrested within 0.02 sec. The signal which was used for the registration of the clamping was found to work quite synchronously with the electromagnet. This was ascertained by simultaneously photographing on a roll-paper camera the movement of the armature of the electromagnet and the movement of the signal.

#### THE BLOOD FLOW THROUGH THE CORONARY ARTERY.

For the success of the experiments, it was imperative to have an accurate measurement of the minute flow through the coronary artery, and an exact knowledge of the phase of the cardiac cycle during which the clamping of the blood supply took place. The cardiac cycle was registered by means of a Wiggers optical manometer (vibration frequency 130–150 per sec.) which was inserted through the left subclavian artery into the aorta, when the experiment was made on the whole animal. In some experiments this was supplemented by an electrocardiogram, for which direct leads were taken from the ventricle. All our observations were made on the circumflex branch of the left coronary artery. The experiments were made on dogs weighing 8–12 kg., anaesthetized with chloralose or sodium luminal. In the whole animal, heparine was used to prevent coagulation. The chest of the experimental animal was opened through a midsternal incision, and artificial respiration was carried out by an

"Ideal" respiratory pump. The coronary artery was either perfused from a reservoir containing blood, which was placed at a suitable height above the animal, or from the aorta of the animal through a connection leading from the subclavian artery to a cannula which was inserted into the peripheral end of the coronary artery. The perfusion from the reservoir (constant pressure perfusion) could be switched over to the perfusion from the aorta (autoperfusion with the pulsatile pressure) by turning a tap with a large bore (Fig. 2). All connections between the perfusion source, whether aorta or reservoir, and the cannula were rigid. The few end-to-end glass connections were made with very inelastic thick pressure tubing. These precautions were especially necessary in the case of the autoperfusion, since otherwise the transmission of the pulse pressure from the aorta to the coronary artery might have been delayed and the cardiac systole would not have coincided with the rise of the blood-pressure in the artery. In consequence of this, the maximal pressure in the coronary artery would have been shifted somewhat towards the diastole. On many occasions we measured simultaneously the blood-pressure in the aorta and in the coronary cannula, and proved to our satisfaction that the rigidity of the apparatus was sufficient to prevent such a displacement. The blood flow through the artery was recorded by means of a small Stolnicov stromuhr of about 10 c.c. capacity. The time taken for a flow of 5 c.c. was measured with an accuracy of half a second, which means that with an average blood flow of 20-25 c.c. per min. the measurements were accurate to about 0.2 c.c.

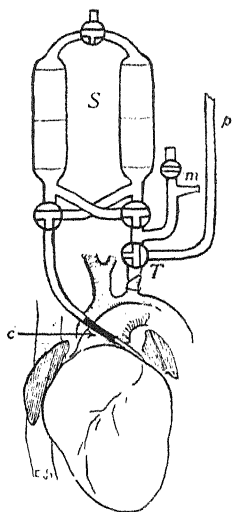


Fig. 2 shows the arrangement of the apparatus. *p* is the connection with the perfusion reservoir containing blood, *m* is the optical manometer, *S* the stromuhr, *c* the place of clamping by the electromagnet, and *T* is a tap by means of which the perfusion of the coronary artery with the constant pressure can be changed to the autoperfusion with the pulsatile aortic pressure. The apparatus is rigid and its taps have a bore of 4 mm. in diameter.

On the way to the coronary artery, and immediately preceding the cannula which was introduced into it, the blood was made to pass through a short piece of thin-walled rubber tubing, the internal diameter of which was 3 mm., while the length of the free part of the rubber tube was equal to 3-4 mm. The rubber tube was placed in a groove underneath the

plunger of the electromagnet which, when periodically activated, clamped the tube in the middle of its free part. The plunger was 2 mm. wide, so that the displacement of fluid in the tube during the clamping was extremely small (about 0.01 c.c.).

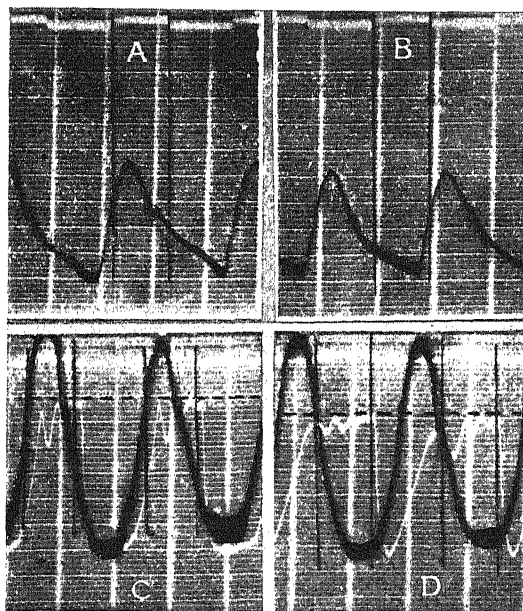


Fig. 3. A and B show the registration of the exact moment and the duration of the coronary clamping in relation to the cardiac cycle. Records of the aortic blood-pressure; the top line is the signal recording the clamping by an upward movement. In A the clamping starts during systole and extends slightly into the diastole; in B it starts in diastole and ends soon after the beginning of systole. Records C and D show a similar experiment, together with a hot-wire registration of the inflow of blood into the perfused coronary artery. The interrupted straight line across the records is the base line for the hot wire. With increase of the blood flow, the shadow of the string moves downwards. The vibrations of the hot-wire record are caused by the impacts of the electromagnet. For other explanations see text. All the tracings in this paper should be read from left to right. Time in 0.2 sec. The time lines in these tracings run somewhat obliquely because of an instrumental error; they are nevertheless accurate.

The heart was driven to beat at a constant rate throughout a whole experiment. Several consecutive measurements of the free blood flow through the coronary artery were made. The clamping circuit was then switched in, and the blood-flow measurements were repeated. In order to know the exact time within the cardiac cycle during which the clamping

took place, the aortic blood-pressure was recorded photographically, together with the movement of the electromagnetic signal. As said before, in some experiments this was supplemented by an electrocardiogram. An example of an actual record is given in Fig. 3A and B. In the first the clamping starts during systole and extends somewhat into the diastole. In the second it starts during diastole and extends into the next systole. The relative position of the clamping period within the cycle was then changed by adjusting the moment at which the driving induction shock was sent into the heart. Measurements of the blood flow were repeated with every new change of the clamping. In between each change the clamping was stopped, and the free flow through the coronary artery was again determined. Usually the procedure was such that three or four measurements of the free flow were interimposed between two or three measurements during the periods of clamping. In some experiments with perfusion of the artery with a constant pressure, a hot-wire registration of the inflow of blood into the artery was also made. An example of such a record is given in Fig. 3C and D, which was obtained from an experiment on the whole animal. In the first record the clamping starts a little after the onset of systole and extends slightly into the diastole. In the second it starts at the dicrotic notch and continues during a long stretch of diastole. The hot-wire record, although distorted by the vibrations caused by the electromagnet, shows that the systolic diminution of the blood flow develops considerably more rapidly in Fig. 3C than in Fig. 3D. In the first case the diminution is started by the systole and accentuated by the clamping of the artery. In the second the diminution is due to the systole alone; the clamping begins at the moment of the minimal blood flow and only serves to prolong this diminution.

Hochrein states that in the whole animal, when the coronary artery is perfused at a constant pressure, the maximal blood flow occurs during systole, which would imply that during systole the heart muscle opposes the minimal resistance to the blood flow. Since, during normal pulsatile aortic perfusion of the artery, the maximal pressure in the coronary artery is synchronous with the maximal pressure in the aorta, one would expect in the whole animal a most vigorous systolic perfusion of the heart. This is the reverse of what we find in the heart-lung preparation [Anrep and Häusler, 1928, 1929] and in the isolated perfused heart [Rössler and Pascual, 1932]. But Hochrein considers that the conditions of the circulation in the heart-lung preparation are so abnormal that the coronary circulation may be entirely changed. Without going into this question any further, we should like to say that it is difficult to under-



stand why a coronary artery, which is perfused at a constant pressure from an outside source, should behave in such a strikingly different manner in the heart-lung preparation and in the whole animal. In our previous experiments we found no such difference. Whether in the heart-lung preparation or in the isolated heart or in the whole animal, the perfused coronary artery had invariably a minimal blood flow during systole

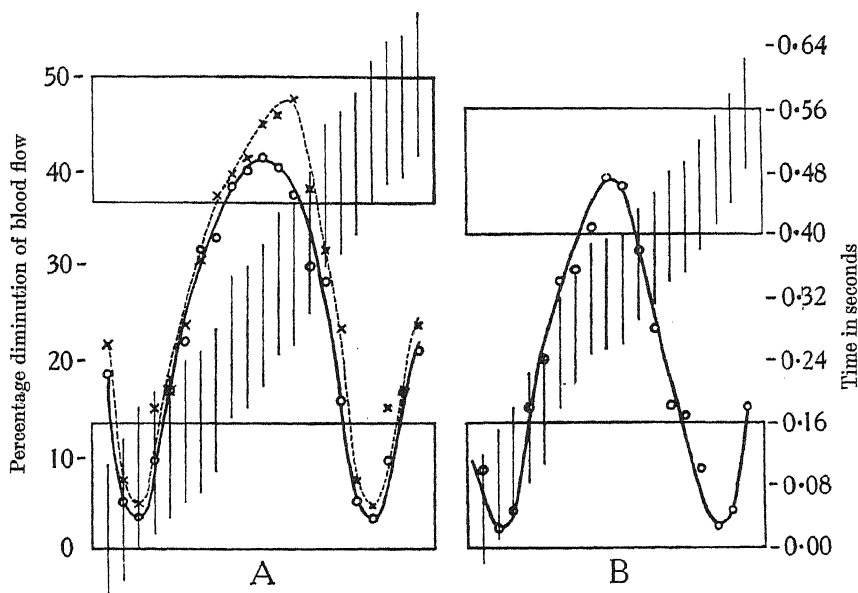


Fig. 4. Percentage diminution of the minute blood flow through the left circumflex coronary artery during short periodical clappings of the artery at various phases of the cardiac cycle. The diagram should be read from below upwards. The two rectangles at the bottom and at the top represent the duration of the systole, and the space between them represents the duration of the diastole. The ascending row of straight lines shows the duration of the clamping and its position within the cycle. A is from an experiment on the heart-lung preparation; B is from an experiment on the whole animal. The dotted line with crosses shows the result of clamping during a constant pressure perfusion; the continuous line with circles shows the result during autoperfusion.

and a maximal during the second half of diastole. However, since the objection has been made, we decided to perform our experiments on the whole animal and to compare the results with those obtained on the heart-lung preparation. The result of two experiments is given in Fig. 4. The left-hand side of the figure shows the effect of clamping of the coronary artery at different periods of the cardiac cycle in a heart-lung

preparation, the right-hand side shows the results obtained on the whole animal.

The figure should be read from below upwards. The width of the two rectangles at the bottom and at the top of each side of the figure represents the average length of systole, while the distance between the rectangles represents the length of diastole. The gradually ascending row of straight lines shows the duration and the position of the clamping of the coronary artery in relation to the cardiac cycle. The part of the lines which lies within the rectangles shows the length of clamping which fell within the systole, and the parts lying between the rectangles within the diastole. In the experiment on the heart-lung preparation, two sets of measurements were made with each new changed position of the clamping, one while the coronary artery was perfused from a reservoir at a constant pressure of 110 mm. Hg, and the other during autoperfusion from the aorta of the same preparation. (The average systolic pressure was 160 and the average diastolic pressure was 100 mm. Hg.) The percentage diminution of the coronary blood flow is shown in the case of the constant pressure perfusion by crosses (dotted line) and in the case of the autoperfusion by circles (continuous line). It can be seen that, with the gradual encroachment of the clamping on the diastole, the effect of it on the blood flow increases. Clamping of the artery during diastole has a conspicuously greater effect than a similar clamping during systole. The clamping during the second half of the diastole has a somewhat greater effect than the clamping during the beginning of the diastole. Fig. 4B shows that there is no difference between the behaviour of the heart in a heart-lung preparation and in the whole animal. The effect of the coronary clamping was found to be in both cases the same in character and magnitude.

In some experiments on the whole animal the clamping of the coronary blood flow at various periods of the cardiac cycle was made during constant pressure perfusion as well as during autoperfusion from the aorta. In other words, the experiment was done exactly in the same way as the one on the heart-lung preparation, the results of which are given in Fig. 4A. The results were the same in the two cases. A large number of observations was made by us on many animals and all cases gave the same results, except that the extent of the effect differed from one animal to another. The greatest diminution of the coronary blood flow during diastolic clamping, which occupied about one-half of the diastole, was 54 p.c. The smallest effect was 28 p.c. Systolic clamping had in some experiments almost no effect on the coronary blood flow, and in others it slightly reduced the flow, usually by not more than about 5 p.c.; only

in one experiment was the diminution of the flow as great as 10 p.c. We found no difference in the result after section of the cervical vagi. From a consideration of these experiments the conclusion seems to be unavoidable that the blood flow through the coronary artery is maximal during diastole and minimal during systole. It also seems obvious that in this respect there is no fundamental difference between the heart-lung preparation and the whole animal and that, disregarding minor points, the circulatory conditions in the perfused and in the autoperfused artery are comparable.

#### THE BLOOD-PRESSURE IN THE CORONARY ARTERY.

Further evidence in favour of this conclusion was obtained by measurements of the blood-pressure in the coronary artery between the place of clamping and the heart. If, during the periods of the systolic clamping, there is little or no inflow of blood into the heart, then the pressure in the cannula, from the beginning of the clamping until the time when the next diastole sets in, must remain approximately at the level at which it was during the moment of clamping. On the other hand, if during the diastolic clamping there is no restriction of the blood flow into the heart muscle, the pressure in the coronary cannula should progressively fall during the whole period of clamping. In order to test this, the coronary cannula was provided with a wide side branch, which was connected to a high-frequency optical manometer. The stromuhr was omitted, so that the whole preparation consisted in the establishment of a communication between the aorta and the coronary cannula. This connection was made entirely rigid except for the 3-4 mm. length of the rubber tube under the plunger of the electromagnet. Thus during the clamping the pressure was measured in the peripheral end of the coronary artery. The cannula carrying the side tube was introduced, as in the blood-flow experiments, into the circumflex branch of the left coronary artery. The heart was driven at a constant rate and the blood-pressure in the artery was recorded during the periods of clamping at various phases of the cardiac cycle. Fig. 5 shows the effect of a purely systolic and of a purely diastolic clamping. The records speak for themselves. During the systolic clamping, not only is there no fall but there is a definite rise of the pressure in the artery; during the diastolic clamping there is a precipitate and conspicuous fall. This effect is equally well marked in an artery which is perfused at a constant pressure and in one which is perfused by the pulsatile aortic pressure. The rise of pressure during systolic clamping

was not observed in all experiments. In many cases, instead of rising, the pressure remained steady at the level at which it was when the clamping took place. It fell abruptly only with the onset of the diastole. In other experiments there was a small fall of pressure during the systolic clamping. This fall, however, was negligible as compared with the huge drop of pressure observed during the diastolic clamping, when the pressure

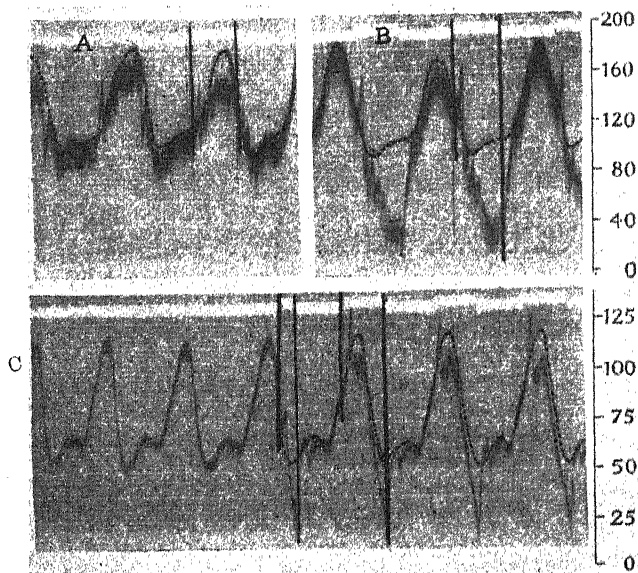


Fig. 5. The effect of clamping of the coronary artery upon the coronary blood-pressure. The top line is the signal recording the clamping; the dotted curves show the blood-pressure before the periodical clamping was begun. Tracings A and B are taken from the same experiment. In A, systolic clamping, in B, diastolic clamping. Tracing C, from another experiment, shows the beginning of a clamping period. The first clamping is very short and purely diastolic. The next clampings start during systole and involve a part of the diastole. Note that the drop of the blood-pressure occurs only during the diastolic part of the clamping. During the systolic clamping the blood-pressure in these two experiments shows a considerable rise.

may fall almost to zero. Only in one experiment was the fall of pressure during the systolic clamping rather considerable, but even here it was still very much smaller than that taking place during the diastolic clamping. In this case a particularly large auricular branch was found to leave the coronary artery just below the nozzle of the cannula.

The difference between the effect of systolic and diastolic clamping is even better observed when the driving of the heart is omitted. The heart

is allowed to beat at its own rhythm, while the clamping is set to work at a rhythm which is slightly faster or slightly slower than the heart rate. The clamping thus gradually shifts with each heart beat from diastole more and more into the systole and then again into the diastole. In this

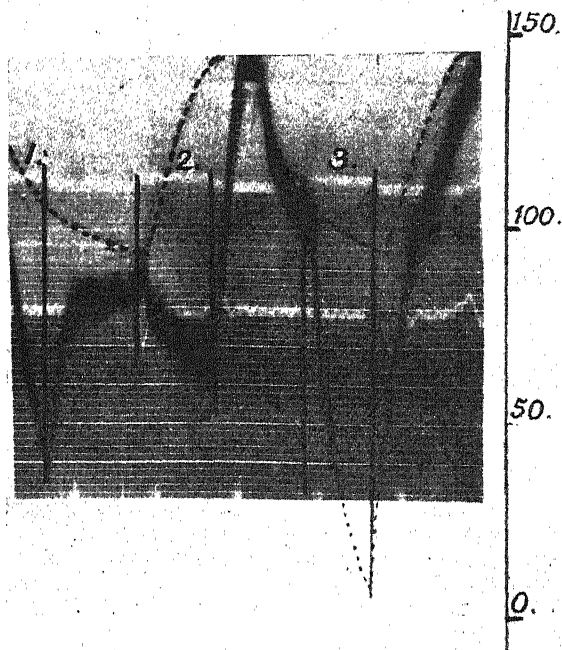


Fig. 6. Whole animal. The effect of two successive clampings of the coronary artery upon the coronary blood-pressure. The dotted curve is a retraced record of the aortic blood-pressure. (1) is the end of a clamping which was partly systolic and partly diastolic, (2) a systolic clamping, (3) a diastolic clamping. The dotted line continuing the drop of pressure in (3) is retraced from the 21st cycle following, in which an exactly similar relative position of the clamping took place and in which the camera was moved so as to include the low pressure levels. An E.C.G. (direct leads from the ventricle) was taken during this experiment. During the systolic clamping the blood-pressure fell by 16 mm. Hg; during the diastolic clamping it fell by 94 mm. Hg, almost reaching zero.

way one gets all the possible combinations in the time relations between the cardiac cycle and the clamping. It would be impossible to reproduce an original record of such an experiment on account of its length. Therefore we give only a small section of a record (see Fig. 6), in which a systolic clamping is followed by a diastolic clamping, and a diagram which is

composed of retraced pressure records obtained in an experiment with autoperfusion on the whole animal (see Fig. 7). We would like again to stress the fact that experiments on the whole animal and on the heart-lung preparation with autoperfusion and with constant pressure perfusion gave the same results.

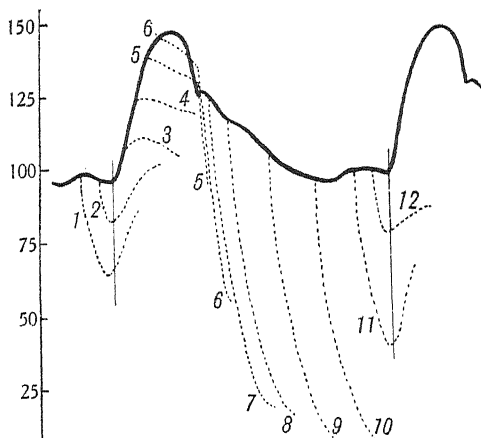


Fig. 7. Retraced superimposed records of changes in the blood-pressure in the coronary artery during its clamping at various phases of the cardiac cycle. All the records were obtained in the same experiment on the whole animal. The continuous curve represents the blood-pressure in the coronary artery when no clamping took place. The dotted lines show the change in the pressure during the period of clamping. The length of clamping in all cases is the same. 1, 2, 11 and 12 begin during diastole and end during systole; 3 and 4 are purely systolic; 5 and 6 begin during systole and end during diastole; 7, 8, 9 and 10 are purely diastolic.

#### SUMMARY AND CONCLUSIONS.

In previous communications from this laboratory it has been shown, by means of hot-wire measurements of the inflow into the perfused left coronary artery, that the heart offers the greatest resistance to the blood flow during the period of systole. These experiments were repeated and no difference was found in this respect between the heart-lung preparation and the whole animal. In further experiments it was shown that, with small differences, the same holds true for a heart in which the coronary artery gets its blood supply from the aorta. The results of the experiments described in this communication lend further support to our former observations by showing that:

(1) Repeated obstructions of the coronary blood flow which occur in diastole conspicuously reduce the blood supply through the artery; on-

the other hand, equal obstructions which occur during systole have almost no effect upon the blood flow. This result demonstrates that the blood supply to the heart during systole is negligible.

(2) Measurements of the blood-pressure between the place of clamping and the heart muscle show that during systolic clamping there is no change or sometimes even a rise of pressure in the coronary artery, while during diastolic clamping there is a precipitate fall of pressure. This result demonstrates that the heart muscle opposes the greatest resistance to the coronary blood supply during systole and the smallest resistance during diastole.

(3) These results were obtained in the heart-lung preparation and in the whole animal, in artificially perfused coronary arteries and in arteries which remained in connection with the aorta.

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## AFFERENT IMPULSES IN THE VAGUS AND THEIR EFFECT ON RESPIRATION.

By E. D. ADRIAN.

*(From the Physiological Laboratory, Cambridge.)*

IN an early paper on sensory nerve impulses [Adrian, 1926] the writer gave a brief account of the discharge in the vagus during respiration. More detailed studies have been made by Keller and Loeser [1929] and lately by Partridge [1933], but neither of them gives records of the discharge in the individual nerve fibres. Without these it is difficult to know whether all the vagal endings are alike, and difficult to compare them with other sense organs. The present work deals with these points and with another question on which there has been some apparent disagreement, that of the existence of sensory endings responding to deflation of the lungs. The results confirm Keller and Loeser in demonstrating such endings, though the deflation needed to excite them can seldom occur in a normal animal. A final section deals with the effects of the vagal discharges on the respiratory centre.

### METHOD.

Most of the experiments on the afferent discharge were made on cats, decerebrated or decapitated under chloroform and left until the effect of the anæsthetic had worn off. Some were made on rabbits anæsthetized with urethane, dial or chloralose, and these anæsthetics were also used in cats when the reflexes were investigated. In all but a few experiments a tracheal cannula was inserted and the movements of the lung were signalled by a rubber tambour leading from a side tube, the main branch being left open or connected temporarily to a 20-litre bottle. A small bottle (3 litres) was used to produce dyspnoea and various gases could be breathed from a Douglas bag. In many experiments artificial respiration was carried out by a pump with a rotary valve producing a known inflation at each stroke (Palmer's "Ideal" model). To study the effect of



increased or diminished external pressure on the thorax the animal was placed in an air-tight tank as in the experiments of Hammouda and Wilson [1932].

Except in the experiments on vagal reflexes the nerve was cut high up in the neck and dissected out for about 4 cm. To obtain the discharge in individual fibres it was subdivided with fine needles after removal of the sheath. Small bundles of nerve fibres are split off the main trunk, and in the cat it is nearly always possible, by further subdivision, to reach a stage at which the impulses form a single series. In the moist atmosphere of the incubator in which the animal is placed the nerve remains in good condition for several hours. Persistent discharges from the cut end seldom occur and can be abolished by further stripping of the sheath. The impulses were recorded in the usual way with a condenser-coupled amplifier, a Matthews oscillograph and a loud speaker. Silver silver-chloride Ringer electrodes were used with worsted leads to the nerve.

#### RESULTS.

A detailed study of the discharge in single fibres was made only in the cat, for the slower rate of breathing makes it easier to analyse the sequence of events than in the rabbit. The general behaviour of the sensory endings is the same in the rabbit, though the existence of a separate cardiac depressor nerve is an important anatomical difference. Since the cat's vagus contains other afferent fibres besides those concerned in respiration it will be convenient to describe first the various types of afferent discharge which are not purely respiratory.

##### *Discharges without the respiratory rhythm.*

The greatest activity in the vagus always occurs at inspiration, and the majority of the fibres which yield measurable action potentials are evidently connected with sense organs which are excited by the expansion of the lungs. But besides these there are groups of impulses occurring at each heart beat, irrespective of the state of the lungs, and there is sometimes a persistent discharge in a few fibres with no sign either of cardiac or respiratory rhythm.

*Persistent discharges.* The records in Fig. 1 C and D show one continuous series of impulses uninfluenced by the expansion of the lungs together with a respiratory discharge of the usual type in several fibres. The former came from an ending near the trachea or lung root: it was increased in frequency by pulling the trachea slightly upwards and

abolished by pressing it downwards (Fig. 1 D). Similar discharges have been found in three preparations, and in some others there have been persistent discharges arising from within the thorax but not affected by the position of the trachea or the expansion of the lungs. Some of them have changed in frequency when the animal was placed on its side, but with others the factors responsible for stimulation have not been made out. The majority of the endings which give persistent discharges are

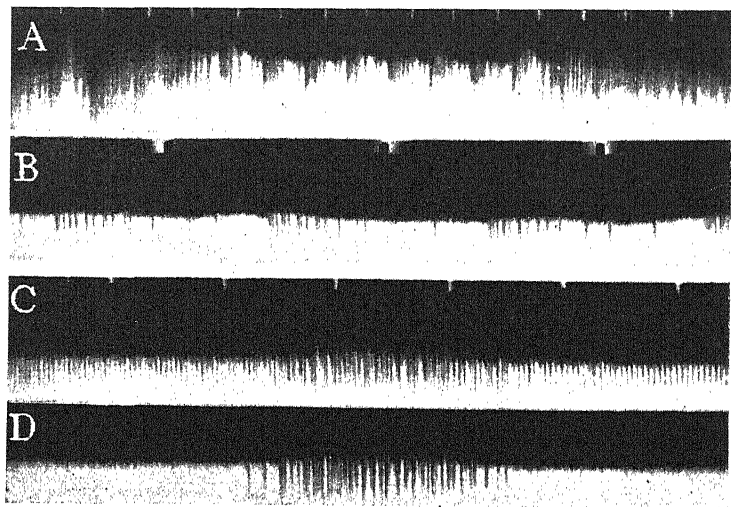


Fig. 1. Records showing afferent discharges in the vagus not influenced by respiration, all from decerebrate cats. A. Preparation with many nerve fibres, some giving cardiac rhythm. The usual respiratory discharge occurs at the beginning and end of the record. B. Record at higher speed showing cardiac discharge in two or three fibres. C. Persistent discharge (frequency 80 per sec.) due to slight traction on trachea. A respiratory discharge occurs in other fibres in the middle of the record. D. Persistent discharge abolished by moving trachea downwards. Time marker (white lines) gives  $\frac{1}{4}$  sec. intervals in these and in all records.

evidently receptors of the slowly adapting or postural type stimulated by mechanical deformation but placed in regions which are not affected by the movements of the heart or lungs.

*Cardiac discharges.* In some cats the cardiac fibres (*i.e.* those giving a pure cardiac rhythm) are grouped into a slender nerve which runs with the vagus but is easily separated from it. In other animals there is no separate sheath, but the cardiac fibres are grouped together, so that if the vagus is split repeatedly cardiac fibres will be present in one or two

of the bundles but absent in the rest. This arrangement is often present in the right vagus although the cardiac fibres are contained in a separate nerve sheath on the left side. Examples of the cardiac type of discharge are shown in Fig. 1 A and B. In Fig. 1 A some of the fibres come from the lung and signal inspiration whilst others give cardiac rhythm. The discharge in each fibre usually consists of three or more impulses at each heart beat: by shunting a fraction of the electrocardiogram into the electrode system it can be shown that the impulses coincide with each systole. The number of impulses at each heart beat is increased by raising the blood-pressure and reduced by lowering it, and in general the discharge agrees very closely with that in the rabbit's cardiac depressor and carotid sinus nerve [cf. Bronk and Stella, 1932].

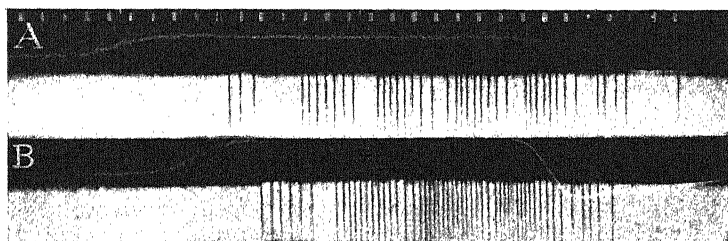


Fig. 2. Record from single fibre of vagus (decerebrate cat) showing discharge with mixed respiratory and cardiac rhythm. A, normal breathing; B, dyspnoea. Signal line moves upwards on inspiration: in these and other records it has been drawn in where it is too faint for reproduction. The duration of each record is 3 secs.

*Discharges with mixed respiratory and cardiac rhythm.* Since the structures near the root of the lung will be deformed by the pulsation of the heart and great vessels as well as by the movements of respiration we might expect to find some endings which are stimulated by both events. These are often found and the type of discharge they give can be seen from the records in Fig. 2. The cardiac rhythm is less constant than the respiratory and may disappear when the position of the animal is changed. The fibres which give this double rhythm form only a small minority of those in the vagus and in many preparations they have not been detected.

*Respiratory discharges: endings stimulated by inflation.*

In natural breathing (cat or rabbit) the frequency of the impulses in the vagus rises to a maximum at inspiration and falls to a minimum as the lungs contract. This is true both for quiet breathing and for extreme

dyspnoea induced by  $\text{CO}_2$ . In the whole nerve there are usually a good many fibres in which the discharge never ceases entirely during the interval between inspirations, though in dyspnoea the number becomes less and less as the deflation of the lungs at expiration becomes more complete. Typical records in which the impulses form a single series are given in Fig. 3 and a record from the rabbit's vagus is included for comparison (Fig. 3 A). Curves of impulse frequency are given in Fig. 4. The frequency rises and falls along a smooth curve as the lungs expand and

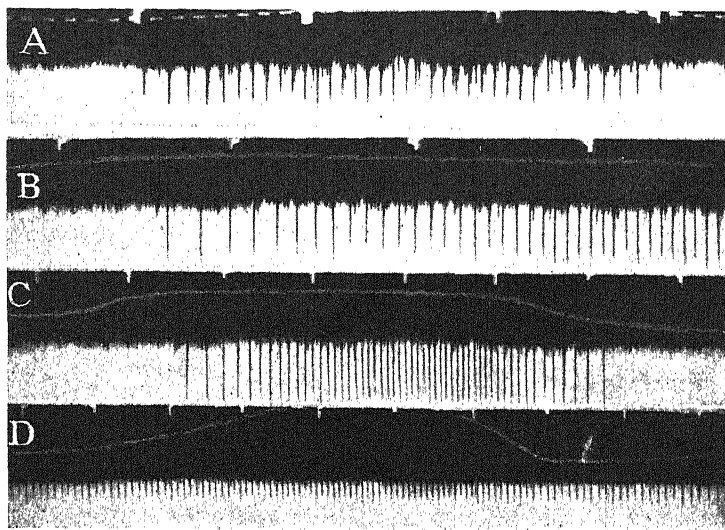


Fig. 3. Normal respiratory discharge in single fibres of the vagus. A, from a rabbit under urethane; B, C and D, from decerebrate cats. White line signals respiration and moves upwards when lungs expand (tambour connected to air reservoir). Time marker gives  $\frac{1}{4}$  sec. intervals.

contract, and the records might well have come from the stretch receptors of a muscle extended and relaxed rhythmically. The likeness remains when the expansion of the lungs is controlled by inflating them artificially. In Fig. 5, for instance, it can be seen that the frequency depends on the degree of inflation and falls off very slowly when the inflation is maintained.

We are dealing therefore with an ending which is like the muscle spindle in having a very slow rate of adaptation to the stimulus. The stimulus is evidently the actual deformation (stretching) of the tissues in which the endings are placed. Air currents are not essential, for the

discharge continues when the lungs are held inflated. The pressure of the air in the alveoli is also immaterial (except in so far as it produces

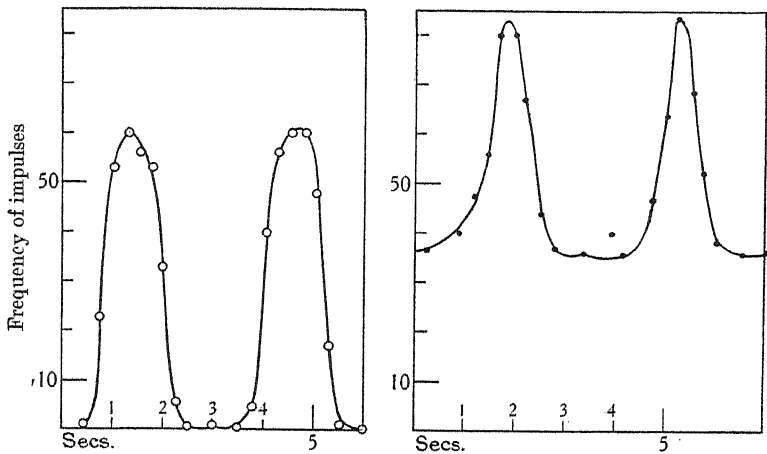


Fig. 4. Frequency of impulses in single fibres of the cat's vagus during normal breathing. Two preparations, both decerebrate.

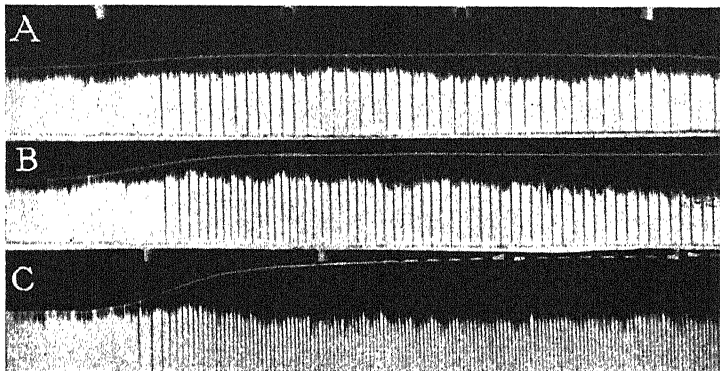


Fig. 5. Spinal cat, single fibre preparation. Inflation of the lungs by pump. Movement of signal line directly proportional to inflation.

A.	Inflation = 65 c.c.	Maximum frequency	80 per sec.
B.	" = 115 c.c.	"	120 "
C.	" = 230 c.c.	"	250 "

deformation), for the discharge is the same for a given degree of expansion whether this is produced by blowing air into the trachea or by the natural movement of respiration.

The relation between the degree of inflation and the frequency of the discharge in single fibres is shown in the curves in Fig. 6. It is approximately linear over the range investigated, and in this it agrees with Partridge's findings for the rabbit's vagus (several fibres). It is scarcely possible, however, to compare the form of the stimulus-frequency relation shown by the vagal endings with that of other sense organs, for in no case can we form more than a very rough estimate of the relation between the stimulus which we measure (volume increase of lung, tension

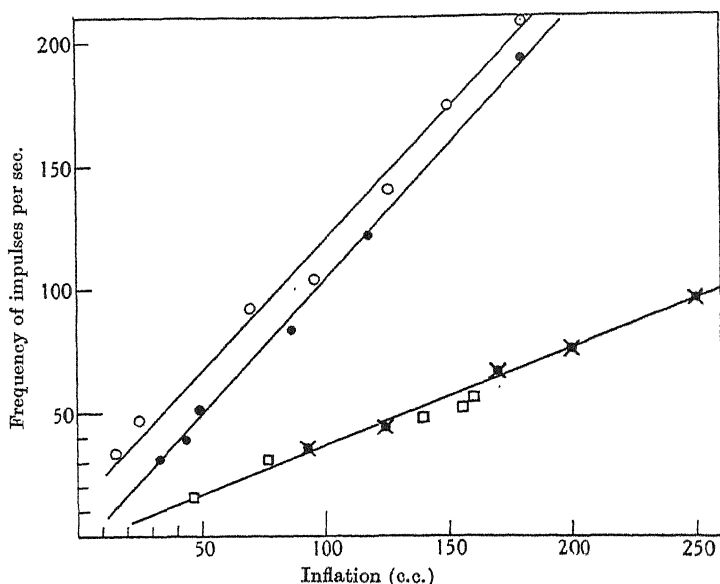


Fig. 6. Maximum discharge frequency in single fibres with varying inflation of lungs. Spinal cats. The two upper curves are from a small animal and show two series of measurements made within 10 min. The lower curve (also two series) is from a larger animal.

on muscle, etc.) and the deformation which it will produce in the sensory endings.

*Adaptation.* Keller and Loeser [1929] state that the discharge in the rabbit's vagus depends on the rate of inflation of the lungs as well as on the final extent, but Partridge [1933] finds no difference in the maximal frequency whether the inflation is slow or rapid. The discrepancy probably arises from differences in the range of stimuli employed, for in single fibres of the cat's vagus there is evidence of adaptation, but with stimuli of moderate intensity the effect is slight. When the degree of inflation

does not produce a frequency higher than about 50 per sec. there is little difference in the maximal frequency caused by an inflation developing in  $\frac{1}{2}$  sec. or 3 sec., and after 10 sec. maintained inflation the frequency has seldom fallen by more than 5 p.c. But with rapid inflations, giving higher frequencies, there is an early decline from the maximum as there is in the discharge from a stretched muscle spindle. Curves illustrating this are given in Fig. 7. It is possible that the initial decline may be due in part to a redistribution of air in the lungs allowing the areas first expanded to contract slightly, but the rate of decline is not appreciably

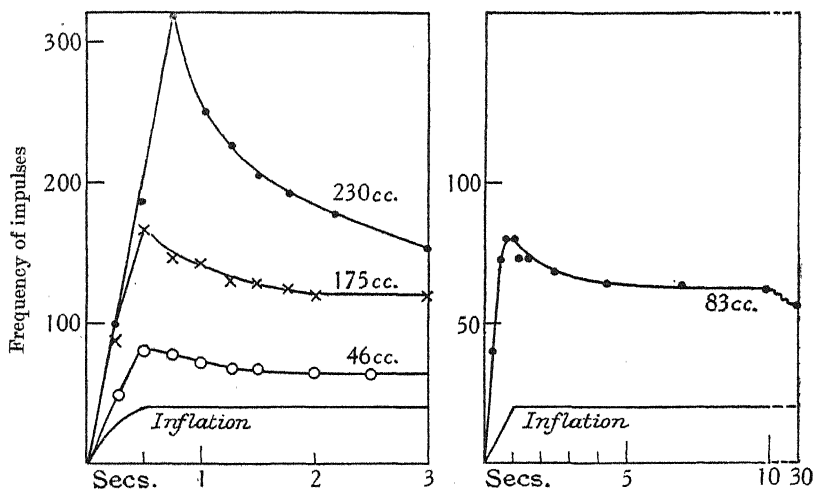


Fig. 7. Frequency curves from single fibres to show adaptation to stimulus. Spinal cat.

changed by binding the abdomen. After the initial decline the rate remains constant or falls slowly provided that the endings are in good condition. The fall is much more rapid when the circulation has failed (see later, p. 341).

*Wedensky effect.* In a few preparations an interesting result has followed from an extreme inflation of the lungs. The frequency rises to 300 per sec. or more and then falls suddenly, the discharge becoming irregular and finally ceasing altogether (Fig. 8 B). If some of the air is allowed to escape, the regular discharge reappears at a frequency in the neighbourhood of 50–100 per sec. This result is obviously an example of the Wedensky effect, closely resembling that found by Tsai [1931] when impulses from a frog's muscle spindle are recorded in a nerve fibre which has been led through a narcotizing chamber. In Tsai's experi-

ments a discharge of low frequency could pass the narcotic block, but if the frequency was raised by increasing the tension on the muscle conduction failed, either partially or completely, to return again when the tension on the muscle (and therefore the frequency of the incoming discharge) was lowered. In the present experiment it is conceivable that the failure occurred in the region of the nerve ending, but more likely that some impairment of conduction was produced in the nerve fibre by the dissection. Even if the fibre escaped all damage, the cut ends of other fibres might affect it by causing an electrotonic block or by raising the concentration of potassium ions etc. in its neighbourhood.

*Variations in threshold, etc.* With the circulation intact and the lungs aerated the vagal endings which signal expansion have all agreed in

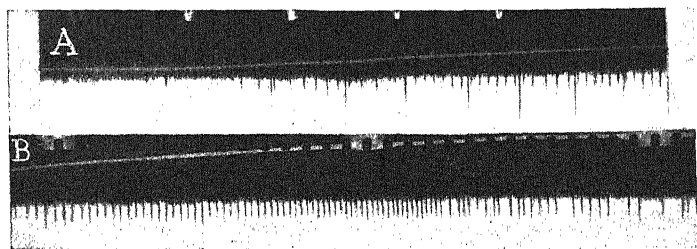


Fig. 8. A. Record from two fibres to show variation in threshold and in discharge frequency for a given stimulus. Spinal cat. The series of small impulses shows a cardiac grouping (as in Fig. 2). B. Wedensky effect. With extreme inflation (250 c.c.) the discharge frequency rises to 300 per sec. and then becomes irregular. Spinal cat.

showing a very slow rate of adaptation. Judged by this criterion all of them are of the same type. There are, however, considerable differences in excitability, or rather in the amount of inflation needed to stimulate the different endings. This may be seen from Fig. 8 A which shows the discharge in two vagal fibres during a gradual inflation.

Whatever its cause, this variation in threshold will evidently increase the range of the signalling mechanism, but it may well depend mainly on the position of the endings, the actual deformation required to excite being the same for all of them. A change in the position of the animal will often produce a change in the response of a single ending to a given inflation. Otherwise the response remains constant over periods of an hour or more.

*The effect of CO<sub>2</sub>, and of circulatory failure.* Bronk and Stella [1932] in their study of the stretch receptors in the carotid sinus have shown



that these endings are very little affected by changes in the acidity or gaseous content of the blood. Their function is to signal the expansion of the artery and nothing else. In the same way the stretch receptors in the lungs are extremely insensitive to environmental changes other than mechanical deformation. This was clearly shown in Head's work [1889] where it was found that inflation of the lungs with  $N_2$  or  $H_2$  produced the usual inhibition of inspiration. Keller and Loeser have found that in the rabbit the substitution of  $N_2$  for air or the addition of 5–10 p.c.  $CO_2$  causes no change in the vagal impulses, and Partridge agrees with them. In the cat (spinal curarized preparation) inflation with air containing 10–12½ p.c.  $CO_2$  for several minutes seems to produce a very slight diminution in the discharge. This is shown by a fall of about 5 p.c. in the total number of impulses at each inflation. The change is scarcely large enough to be outside the range of error: it is certainly too small to be the basis of any considerable change in the depth of breathing. But as Keller and Loeser have pointed out, in the intact animal  $CO_2$  may affect the vagal discharge indirectly by causing alterations in the calibre of the bronchioles.

Failure of the oxygen supply is another factor which has remarkably little effect on the vagal endings. The air may be shut off or nitrogen substituted for it until the heart has ceased to beat, without causing any decided change in the response to a measured inflation. In mammalian muscle Matthews [1931] has found that, if the effect of the anæsthetic has worn off, arrest of the circulation leads invariably to a spontaneous discharge of high frequency from all the sensory endings. With the vagal endings the only suggestion of this is a persistent discharge of low frequency (20 per sec.) which develops in some of the fibres when the lungs have been inflated rhythmically with nitrogen. The difference is easily explained, for Matthews found that the discharge from the muscle receptors was caused or greatly favoured by something produced in the muscle fibres and produced much more rapidly if they are made to contract. Since few of the vagal endings are likely to be exposed to metabolites from muscle there is no reason to expect great spontaneous activity in many of them.

The vagal endings cannot, of course, survive indefinitely after the failure of the oxygen supply and they become inactive within half an hour to an hour after the heart has stopped. The first sign of deterioration is a more rapid decline in the frequency of the discharge under a constant stimulus. A series of curves illustrating this are given in Fig. 9. They show the same change as Bronk's curves [1929] for the effect of nitrogen



and within a minute or two it fails completely. It must be remembered, however, that during this time the lungs are still being inflated with undiminished force by the pump: the conditions are therefore quite unlike those in a patient breathing naturally. If the chloroform is turned off within a minute or so after complete failure there is an immediate recovery of the vagal discharge and within another minute it is back at its initial value. The rapid failure and recovery are illustrated in the records given in Fig. 10 which show a return almost to normal within

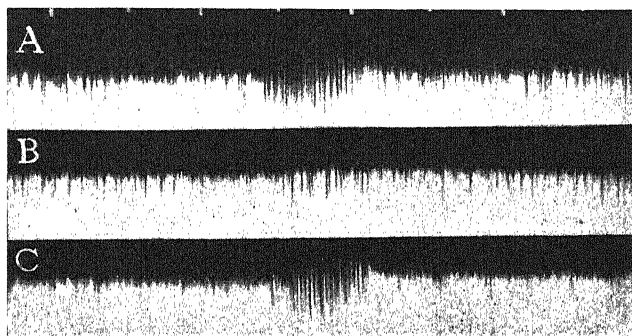


Fig. 10. Rapid failure with strong chloroform vapour (5 p.c.) and rapid recovery with air. Spinal cat. Lungs inflated by pump. A, with air; B, 70 sec. after chloroform has been turned on; C, recovery after 10 sec. inflation with air.

10 sec. Attempts to maintain a partial anaesthesia of the vagal endings were unsuccessful. The lower concentrations of chloroform were too weak to affect them (though amply strong enough to affect the central nervous system) and the higher caused a rapid failure. This may be due to the rate at which the chloroform is absorbed by the blood stream, but it is difficult to produce a graded anaesthesia of a nerve trunk with chloroform vapour, and the action on the vagal endings may be equally abrupt.

The practical outcome of these experiments is to show that the strength of chloroform normally administered to a patient (*i.e.* below about 2.5 p.c.) is without effect on the endings in the lung which respond to mechanical stimulation. Very high percentages may affect them, but a few inflations with air should be enough to give complete recovery.

*Endings stimulated by deflation of the lungs.*

In the brief account given by the writer in 1926, it was stated that in normal breathing there is no sign of a renewed discharge of impulses at the moment when the lungs are most deflated, and that forcible deflation

of the lungs in a spinal cat does not give rise to a discharge. The forcible deflation referred to was that produced by compressing the thorax by hand, and this, in the cat, is not enough to stimulate the endings which respond to deflation. But Keller and Loeser showed that suction of air from the lungs undoubtedly gives rise to a discharge of nerve impulses. Their experiments were made on rabbits, and in the present work it has been found that the result is equally true of the cat: forcible deflation of this kind does produce a discharge.

In the cat when the thorax is opened the discharge can only be produced by deflating the lungs completely. It then varies rhythmically as each pulsation of the heart moves the collapsed lung. When the thorax is intact the discharge is usually continuous as long as the suction is maintained (Fig. 11), though in some preparations there has been only a short outburst at the beginning. From this it appears that some of the nerve endings are of the more rapidly adapting type, but the majority

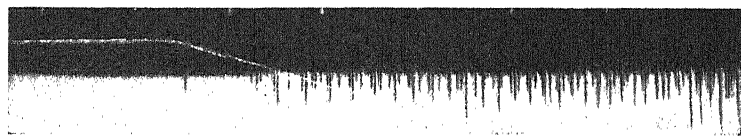


Fig. 11. Afferent discharge produced in several fibres by suction of air from the lungs. Spinal cat.

are like those responding at inspiration. It is possible and indeed likely that some of the endings which react to suction are those which react normally to inflation, for both inflation and extreme deflation might stretch the tissues in which they lie. But there is no doubt that deflation calls a new set of endings into play as well. When only a few fibres are in action the impulses due to inflation and suction can often be distinguished by their size and form; also suction of air from the lungs has a specific effect on the respiratory centre. If the vagi are intact it causes an immediate contraction of the muscles which produce inspiration, whereas an inflation of the lungs has the reverse effect, since it inhibits inspiration. Thus the endings concerned in the response to extreme deflation must form part of a distinct nervous mechanism, though it is a mechanism which must be very rarely called into play.

The rabbit agrees with the cat in showing no expiratory discharge in normal breathing or in dyspnoea [Keller and Loeser, Partridge], but it differs from the cat in that it is usually possible to produce one by increasing the external air pressure on the thorax and abdomen so as to

assist the collapse of the lungs. The animal is placed in a closed chamber with the tracheal tube communicating with the air outside, the arrangement being a copy of that used by Hammouda and Wilson in their study of the vagal reflexes. Raising the air pressure in the chamber assists the collapse of the thorax at expiration and hinders the expansion at inspiration. The records in Fig. 12 were made from the vagus of a rabbit under these conditions, and it will be seen that with increased external pressure the usual discharge at inspiration is greatly reduced and in its place a definite expiratory discharge appears. In the cat this method has always failed to give an expiratory discharge, owing presumably to the greater strength of the body walls.

Whether the endings which are stimulated by collapse play much part in normal breathing is exceedingly doubtful. In the decerebrate or

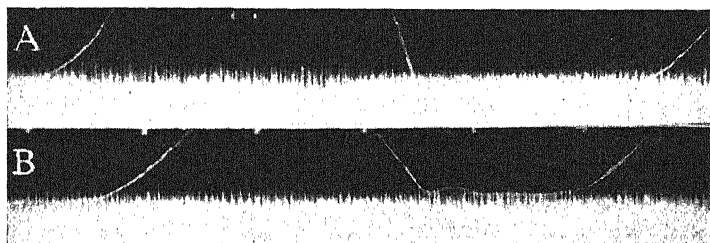


Fig. 12. Afferent discharge at expiration produced by increased external pressure in the rabbit. Rabbit (under urethane) in air-tight chamber with trachea tube leading outside. A. Normal extra-thoracic pressure. Usual inspiratory discharge. B. Increased extra-thoracic pressure. Expiratory discharge and quickened breathing.

anæsthetized animal they play none at all except in conditions which are definitely abnormal. The failure to record a discharge at expiration has not been due to lack of activity in the expiratory muscles, for these have often contracted vigorously. It is possible that the forcible deflation occurring in extreme dyspnoea might stimulate the expiratory endings, but so far this has not been observed. On the other hand it is highly probable that these endings may come into play in various pathological conditions, *e.g.* when portions of the lungs are solidified or tied to the chest wall by adhesions.

It should be noted here that suction of air from the lungs (or breathing into a reservoir containing air at reduced pressure) will not necessarily have the same effects as increased pressure applied outside the thorax. Suction may well stretch some of the endings which are normally stretched at inspiration, but increased external pressure is unlikely to

do so. This difference may explain some of the divergent results which have been reported with the two procedures [cf. Hammouda and Wilson, 1932; Creed and Hertz, 1933].

*The stretch receptors and the respiratory centre.*

So much has been written about the action of the vagus in respiration that a brief discussion cannot hope to do justice to earlier work. It can only be excused by the fact that a more detailed knowledge of the afferent discharge gives a better foundation for analysing the vagal effect. We know that inflation of the lungs will produce a sustained discharge from the stretch receptors and that the deflation normally occurring will merely put an end to this discharge, though suction of air from the lungs will excite another set of endings. In normal conditions, therefore, the effect of the vagus must depend mainly, if not entirely, on the impulses from the stretch receptors. Persistent discharges unaffected by lung movements have been found in a few preparations and there is always the possibility of action currents too small to detect. But the dominant activity in the vagus is that due to the stretch receptors, and it is worth considering whether their discharges are enough to account for the whole of the vagal effect. In particular, can they account for the fact that more rapid breathing occurs when the vagi are intact than when they are cut? The messages from the stretch receptors have an inhibitory rather than an excitatory effect. Why should more rapid breathing be possible in their presence than in their absence?

The question was discussed by Hammouda and Wilson [1932]. Their evidence, based on the reactions to altered extra-thoracic pressure, led them to a picture of the sensory mechanism of the vagus which agrees in the main with that given here. But to explain the slowing of breathing on division of the vagi they suggest that the vagus has a double influence on the respiratory centre, an inhibitory influence varying in intensity with the lung volume, and besides this an influence which maintains the tone of the centre and augments the frequency of the discharge. The latter is considered to remain in general at a constant level of intensity unaffected by changes in the tension of the lung tissues.

Such an influence might be exerted by impulses in nerve fibres too small to give measurable action currents. It is quite possible, however, that the one sensory mechanism is enough: that the quickening of the rhythm is due to impulses from the stretch receptors and results from their inhibiting each period of inspiratory activity in the brain stem. An explanation on these lines was in fact given in the classical paper

published by Henry Head from Hering's laboratory in 1889, though it appeared then that the expiratory endings might play some part in the effect.

Later evidence has confirmed the main points in Head's theory. In the first place there is now no reason to doubt the power of automatic action by the respiratory centre. We are dealing with a group of neurones in the brain stem which tend to discharge periodically without the need for any periodic inflow of afferent impulses. Winterstein [1911] has shown that a recurrent discharge of motor impulses takes place in the phrenic nerve although the vagi have been cut and all movement has been paralysed by curare (and this has been confirmed in the experiments of Adrian, Bronk and Phillips [1932] as well as in the present work). During complete motor paralysis and a temporary pause in the artificial ventilation afferent impulses will still reach the central nervous system from pressure receptors, etc., but there will be no waxing and waning of the incoming stream to determine the period of the phrenic discharge. This is slower than that of normal breathing but no slower than that of breathing after division of the vagi. Thus the respiratory centre (or some part of the brain stem apparatus concerned in respiration) must be capable of beating with its own inherent rhythm. Its cells cannot be regarded as passive agents controlled entirely by afferent messages, for internal changes must be constantly taking place tending to produce a recurrent development of the active state.

Without the vagi the centre usually beats at a slow rate and only small variations of rhythm are possible. The messages from the stretch receptors change this spontaneous beat to the more rapid and flexible rhythm of normal breathing. They are inhibitory, but they can influence the rhythm in either direction because inhibition of the phase of activity will shorten this part of the cycle and tend to hasten its return, but inhibition during the phase of rebuilding will delay it. The dual influence discussed by Hammouda and Wilson depends, on this view, not on a dual afferent mechanism, but on the dual effect of inhibition on the respiratory centre. In Head's words the vagus inhibits inspiratory activity but at the same time raises the vitality of the centre by causing this activity to accumulate.

The maximum inhibitory effect occurs during the phase of activity and therefore the rhythm is usually quicker with intact than with cut vagi. The motor discharge will be curtailed, there will have been a smaller expenditure of active material and it is reasonable to suppose that less time will be taken in preparing for a fresh outburst. Thus a

series of brief inhibitions at each expansion of the lungs should give a cycle consisting of brief motor discharges repeated at short intervals; whereas the absence of all inhibition should give longer discharges spaced at greater intervals. This is the characteristic effect of division of the vagi, for the slowing is usually due to a prolongation of both the active and inactive phases of the cycle.

Fig. 13 illustrates the slowing of the rhythm due to the abolition of the vagal control and the quickening due to recurrent inhibition. The

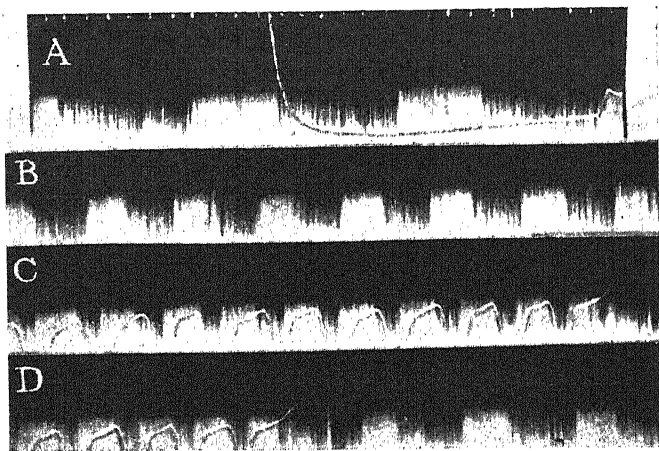


Fig. 13. Discharge of motor impulses in the top root of the phrenic nerve cut distally. Decerebrate cat. All records at same speed. A. Both vagi blocked with novocaine. Slow rhythm (27 per min.) unaffected by sudden inflation of the lungs. Downward movement of signal marks inflation. B. Vagi intact. Normal breathing 58 per min. C. Vagi intact. Periodic inflation of lungs timed so as to inhibit each motor discharge. Downstroke of signal shows inflation. The rate increases to 90 per min. D. After 11 sec. the periodic inflation is stopped. The rate falls at once to 48 per min. Time marker (top record) gives  $\frac{1}{4}$  sec. intervals.

records show the discharge of motor impulses in the top root of the phrenic in a decerebrate cat. When the vagi have been temporarily blocked with novocaine the motor discharges recur at 25 per min., each lasts for  $1\frac{1}{2}$  sec. and the cycle is quite unaffected by inflation of the lungs. With intact vagi the inhibition normally occurring at each breath cuts short the motor discharge after it has lasted only  $\frac{1}{2}$  sec. The rate of breathing is now 58 per min. By blowing air into the lungs at each inspiration the inhibitions are increased in intensity, the motor discharges are shortened still further and the rate rises to 90 per min., falling at once to 48 as soon as the inflations are discontinued. It will



be seen that both parts of the cycle are affected: the change in rate is due mainly to the shortening of the phase of activity, but there is some reduction in the interval between the end of one discharge and the beginning of the next. This is shown more clearly in the records in Fig. 14 which are made at higher speed.

This is little more than an example of the well-known fact that in an animal under artificial respiration the rhythm of the active movements of the diaphragm may come into phase with that of the pump. It shows that an increase in the inhibitory message at each inspiration can produce a quicker rhythm: it is therefore unnecessary to suppose that the vagus exercises its tonic effect on the centre through a distinct set of nerve fibres.

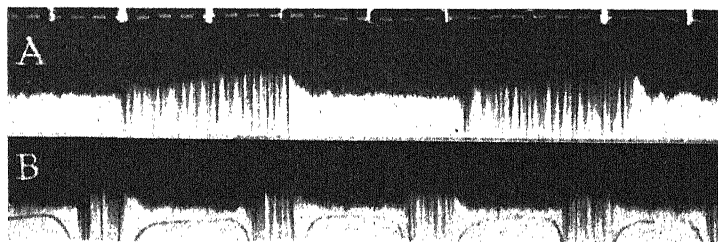


Fig. 14. Same experiment as Fig. 13. Records at higher speed (time marker gives  $\frac{1}{4}$  sec.). Signal line drawn in. A. Normal discharge of motor impulses in phrenic root. B. Effect of periodic inflations. Both active and inactive phases are shortened.

The opposite effect will be produced if the inhibitory message acts throughout the period when the centre is recharging, for this will tend to delay the appearance of a fresh discharge. Thus the rhythm, in so far as it is determined by the stretch receptors, will be most rapid when the inhibitory effect rises sharply at each inspiration and falls to zero at expiration (Fig. 15 A) and slowest when the rise is less pronounced and there is some persistent inhibition throughout the cycle (Fig. 15 B). The frequency of the afferent discharge varies with the lung volume (p. 338). Therefore, if we can assume that the inhibitory effect does not much outlast the afferent discharge, the curves in Fig. 15 might relate to lung volumes instead of to inhibitions and the quickest rhythms should occur when the lung volume changes as in Fig. 15 A. The inhibition does no doubt outlast the afferent discharge, but records such as those in Fig. 13 show that it can rise and subside rapidly enough to affect only a limited part of the cycle. The conclusion given above is best illustrated by two

examples, the change in rhythm produced by altering the external pressure on the thorax and that occurring in  $\text{CO}_2$  dyspnoea.

*Increased pressure on the thorax.* The effects of this have been studied in detail by Hammouda and Wilson, using the air-chamber method. They worked on dogs and found that an increase or reduction of pressure in the chamber caused an immediate quickening or slowing of the breathing. The changes only occur with intact vagi and they occur before the change in ventilation can have any effect.

A number of experiments have been made on cats by the same method, the afferent discharges in the vagi being sampled by recording the impulses in a few fibres slit from the side of the nerve. The earlier

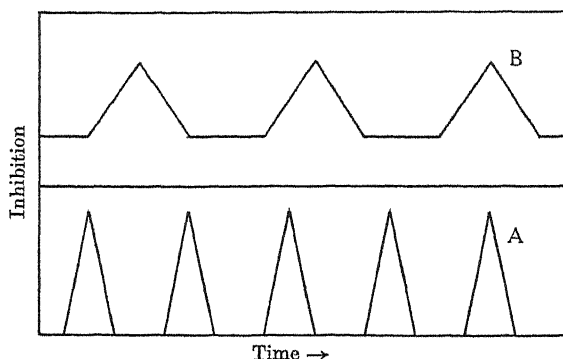


Fig. 15. B. Rise and fall of inhibition likely to give slow breathing.

A. Rise and fall of inhibition likely to give rapid breathing.

experiments failed to show the reaction to increased pressure and it was thought that its occurrence in other animals might depend on the stimulation of expiratory endings. But in later work, with chloralose as anæsthetic, the reaction was found in the cat, although there was never any sign of an afferent discharge produced by the deflation of the lungs. Fig. 16 gives typical curves showing the frequency of the vagal impulses (in several fibres) just before and just after the pressure in the chamber is raised. As might be expected, the whole frequency range is lowered when the thorax is reduced in volume, and in particular the discharge is much briefer and sinks to zero between each inspiration instead of remaining at a moderate level.

The quickening of the breathing is shown in the curves. It is evidently due to the reduced inhibition, but on the scheme outlined above it must be due not so much to the reduction in the peak of the inhibitory

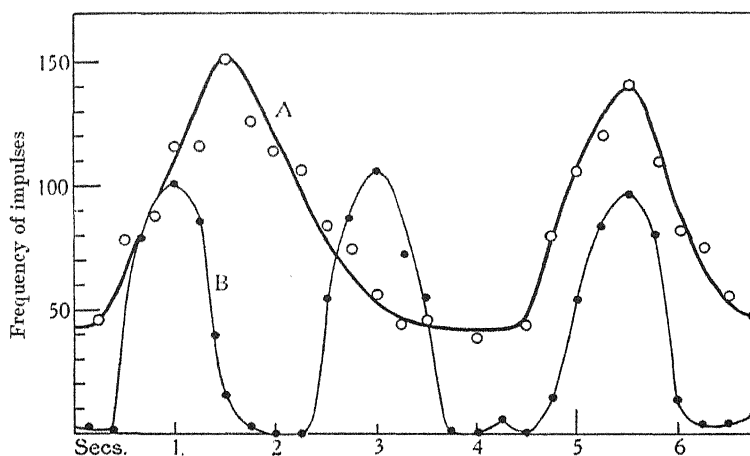


Fig. 16. Afferent vagal discharges in cat anesthetized with chloralose. One vagus intact. Frequency curves (preparation of several fibres) showing the effect of increased extra-thoracic pressure on the discharge and on the rate of breathing. Curve *A*, normal extra-thoracic pressure. Rate 15 per min. Curve *B*, 5 mm. Hg positive pressure outside thorax. Rate 27 per min.

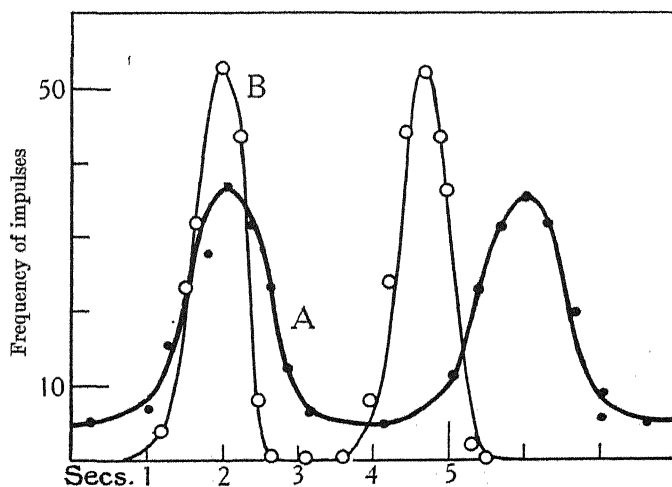


Fig. 17. Afferent vagal discharge in decerebrate cat showing the effect of dyspnoea due to rebreathing. One vagus intact. *A*. Normal, rate 15 per min. *B*. Dyspnoea, rate 22 per min. The impulses have been counted in two inspirations only.

effect as to its reduction in the later part of the cycle. By recording the action currents in the intercostals it can be shown that the motor discharges are more powerful and of slightly longer duration when the pressure is increased. This would naturally follow from the smaller inhibitory discharge at each inspiration. The greater motor discharge should tend if anything to lengthen the period required for rebuilding, but as there is less residue of inhibition during this phase the threshold for the discharge will be lower and will be reached earlier. If this is the factor which determines the increase in rate we ought to find that increased pressure on the thorax will have the greatest effect when there is a considerable background of persistent inhibition. This is certainly the case, for the preparations which have shown the quickened rhythm have been those in which the vagal discharge has normally remained at a fairly high level during expiration. Some tonic contraction of the diaphragm and intercostals (as often occurs with chloralose) favours the reaction, since it keeps the lungs slightly expanded; where such tone is absent there is often no increase in rate with increased intrathoracic pressure though there is a considerable slowing when the pressure is reduced. A reaction of this kind is usually found when dial or urethane are used as anæsthetic.

*Change of rate in dyspnœa.* The quickening of breathing in  $\text{CO}_2$  dyspnœa can be explained on the same lines. In the decerebrate cat with vagi cut the rate rarely changes by as much as 25 p.c. when the animal breathes into a closed space. With the vagi intact the rate may be more than doubled. Curves showing the frequency of the vagal impulses are given in Fig. 17, though, as before, the changes in lung volume might be substituted for them. Under the increased  $\text{CO}_2$  there is (a) a steeper rise to a higher maximum frequency at inspiration, due to the more forcible movement, and (b) a more sudden fall to a lower minimum at expiration, due to the greater emptying of the lungs. If it had much after effect the greater vagal discharge at inspiration might tend to slow the rhythm by interfering with the recovery phase, but the cutting short of activity and the absence of any vagal discharge during recovery will both tend to quicken the rhythm. By exposing the thorax to reduced external pressure (Hammouda and Wilson's method) the greater collapse of the lungs can be prevented and the vagal discharge at expiration restored to its former value. This causes an immediate slowing of the rate, though as asphyxia supervenes the effect is less and less marked, owing, presumably, to the rapid building up of active material in the centre.

*The stretch receptors and the expiratory muscles.*

The emptying of the lungs may be an active as well as a passive process and the afferent discharge in the vagus may influence expiration as well as inspiration. It is a simple matter to record the action currents of the expiratory muscles with hypodermic needle electrodes, for these allow us to lead from the individual motor units in each muscle without dissection of the chest wall and without danger of interference from neighbouring muscles. By this method it can be seen that in cats and

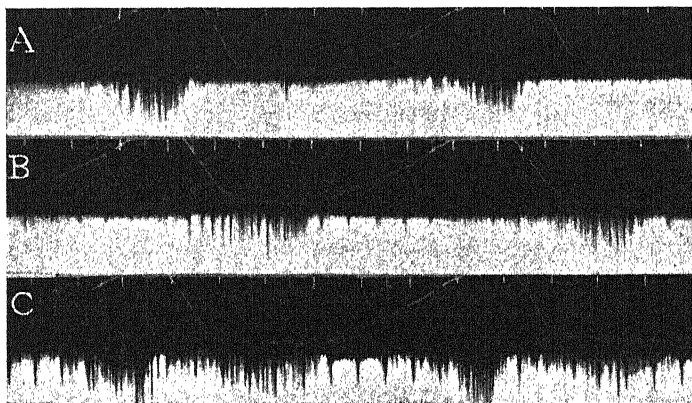


Fig. 18. Records from the external intercostal muscles (inspiratory) and the triangularis sterni (expiratory) made with hypodermic needle electrodes in a cat anaesthetized with chloralose. Signal moves upwards when lungs expand. A. External intercostal. B. Triangularis sterni. C. Both needles in parallel to show time relations of inspiratory and expiratory contraction. The regular excursions are due to the electrocardiogram.

rabbits under chloralose (and lying on the back or side) the triangularis sterni and the internal intercostals are regularly in action during the expiratory phase of the cycle. In decerebrate preparations and in animals under dial and urethane they are occasionally in action, in deep chloroform anaesthesia never. When they are not in action during quiet breathing they are not usually brought into action by moderate degree of dyspnoea.

Records from a cat under chloralose are given in Fig. 18. Two hypodermic electrodes were used, one in the external intercostal muscle and one with its point a few mm. deeper so that it led from the triangularis sterni. In record C both needles were connected in parallel with the

amplifier so as to show the time relations of the two discharges more clearly. The respiratory signal records the pressure change in a 20-litre bottle connected to the trachea tube. In these records the expiratory discharge fills up most of the gap between inspirations. If chloroform is given in addition to the chloralose the discharge becomes progressively shorter and is limited to the period when the deflation is at a maximum. Eventually it disappears, and in very deep anaesthesia the contraction of the external intercostals also ceases leaving only the diaphragm in action.

The periodic contraction of the expiratory muscles, when it occurs with the vagi intact, continues like that of the inspiratory muscles after the vagi are divided. The expiratory activity preserves its phase relationship to the inspiratory and both are unaffected by the state of expansion

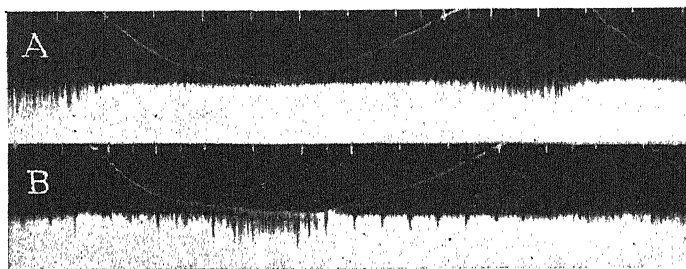


Fig. 19. Same preparation as Fig. 18 after section of both vagi. A. External intercostal. B. Triangularis sterni. Inflation or deflation of the lungs has now no effect on either discharge.

of the chest (Fig. 19). It is probable, therefore, that the expiratory discharge is as much a product of the automatic beating of the respiratory centre as the inspiratory. The nerve cells responsible for it are so linked to the nerve cells responsible for inspiration that the two groups cannot come into action simultaneously and normally the expiratory activity occurs as an after effect of the inspiratory. But it can also be made to occur independently by an afferent discharge from the stretch receptors. There is some variation in this respect between one preparation and another. In some an inflation of the lungs causes an immediate expiratory contraction even though it is timed so that the normal inspiratory contraction is completely suppressed. In others the expiratory contraction develops more slowly and an inflation of the lungs is a less potent stimulus than a period of inspiratory activity.

In his account of respiration in animals after various operations on the brain stem Lumsden [1923] states that electrical stimulation of the

vagus inhibits expiratory contractions. This may well be due to the effect of the nerve fibres coming from the endings which are excited by suction of air from the lungs. These cause an immediate reflex inspiration and an inhibition of the expiratory contraction, and on electrical stimulation of the vagus their effect may outweigh that of the fibres from the stretch receptors. The latter certainly favour expiratory as they inhibit inspiratory activity.

As far as the vagal control of breathing is concerned, the action of the expiratory muscles can be regarded merely as an additional factor tending to reverse the effect of inflation. In so far as it secures a smaller lung volume at expiration the contraction of these muscles will help to establish the type of vagal discharge which promotes rapid breathing. But in animals under chloralose there is often some tonic contraction of the diaphragm to oppose deflation. Thus the breathing may be no faster than it is in animals with no expiratory activity but more complete muscular relaxation.

#### DISCUSSION.

As usual it is easier to reach definite conclusions about the sensory mechanism than about its action on the central nervous system. The main group of sense organs in the lung behaves no differently from the muscle spindles or stretch receptors in other parts of the body. They give the usual serial discharge of impulses when they are stretched, they become very slowly adapted to the stimulus, they are relatively unaffected by anæsthetics, lack of oxygen, etc. Their function is to signal the volume of the lungs at each moment and they continue to do this for half an hour or more after the heart has stopped beating. Since the volume of the lung changes relatively slowly, the endings must be of the slowly adapting or postural type. The only evidence of rapidly adapting endings supplied by the vagus is the occurrence of an initial brief discharge in some preparations when the lungs are collapsed by suction, but these have not yet been analysed in records made from a few nerve fibres.

The stretch receptors in the lung resemble those in muscle in their effect on the central nervous system. Both influence it to cut short the movement which has stimulated them, and by so doing prevent the inconvenience or damage which might come from unrestrained motor activity. But the lungs must be adequately ventilated, and if the range of movement is restricted the movements must succeed one another at shorter intervals. Without the vagal mechanism the respiratory centre (in the conditions of these experiments) slowly charges and discharges

itself with little variation of rhythm. The impulses from the stretch receptors quicken the rhythm by cutting short the discharge and so hastening the recharging process. They are inhibitory, but they seem to act mainly by raising the discharge threshold, not by preventing the accumulation of active material.

This view need not be further elaborated since it was stated with more detailed evidence in Head's paper. It postulates a type of inhibition which seems to agree in general with the results of Eccles and Sherrington on the limb reflexes. It is natural to compare the inhibition of the respiratory beat with that of the heart beat, and at first sight the results seem to differ radically, for the respiratory beat is quickened and the heart beat slowed. But the former is quickened by inhibition confined more or less to each period of activity; it is slowed if the inhibitory discharge fills up the whole cycle. When the heart is slowed by electrical stimulation of the vagus it is exposed to a steady inhibition comparable to that produced on the respiratory centre by holding the lungs inflated. The slow subsidence of the effect from single stimuli [Brown, Eccles and Hoff, 1932] makes it unlikely that the inhibition of the heart could be made to fluctuate rapidly enough in relation to the cycle to establish a fair parallel between the two cases.

Throughout the preceding discussion the term respiratory centre has been used as a name for that part of the central nervous system in which the rhythmic activity takes origin. Where this occurs is a matter for studies such as those of Lumsden [1923] and does not concern us here. Slow potential changes in phase with respiration can be detected in the brain stem of the rabbit and seem to resemble those found in the isolated brain stem of the goldfish by Adrian and Buytendijk [1930]. In the mammal, however, the brain stem cannot be isolated and it is a much more difficult matter to analyse the waves and to make sure that they are not artefacts due to movement of fluid, etc.

#### SUMMARY.

The action of the vagus in respiration has been studied in the cat by recording the impulses in single afferent fibres.

1. Some of the discharges come from end organs which are not affected by lung movement, *e.g.* from end organs in relation to the heart and to the trachea.

2. Of the end organs which respond to lung movement and give measurable action currents, the only kind in action normally is that stimulated by expansion of the lung.



3. These end organs behave like the muscle spindles and stretch receptors in other parts of the body. They become very slowly adapted to the stimulus. In normal breathing the maximum frequency of the discharge in single fibres varies from 50 to 100 per sec., though rates as high as 300 per sec. are produced by extreme inflation. With moderate inflations the frequency varies directly with the lung volume.

4. Carbon dioxide in amounts higher than 10 p.c. produces a very slight diminution in the discharge to a test inflation, but the effect is not outside the range of error. Ventilation with nitrogen and failure of the circulation have no immediate effect. After 10–20 min. the discharge to a constant stimulus declines more rapidly, but there may still be some response an hour after the heart has stopped beating.

5. Chloroform vapour in amounts of 2·5 p.c. or less has no obvious effect on the response of the end organs. Higher proportions produce a rapid failure, but there is a rapid recovery as soon as the lungs are inflated with air.

6. Suction of air from the lungs stimulates a fresh set of end organs. These may come into action in pathological conditions, but they are not stimulated by the normal movement of the lung and there is no evidence of their activity in moderate dyspnoea. In the rabbit they are stimulated at expiration when the extra-thoracic pressure is increased.

7. The effect of the vagus on respiration must be due mainly to the impulses from the stretch receptors in the lung. These have an inhibitory effect on the respiratory centre, but the inhibition occurs mainly during the phase of activity. Periodic inhibitions may therefore quicken the rate of breathing, as Head suggested, by cutting short the motor discharge and so hastening recovery.

8. The expiratory muscles (triangularis sterni and internal intercostals) contract regularly in cats and rabbits anaesthetized with chloralose. They are sometimes in action in decerebrate cats but never in cats under deep chloroform anaesthesia. In chloralosed animals the expiratory muscles remain in action after section of both vagi.

9. The afferent control of the respiratory movements is discussed.

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# THE RENAL ELIMINATION OF INJECTED UREA AND CREATININE.

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## INTRODUCTION.

THE subject of the present paper is the experimental determination of the renal extraction ratios for injected creatinine and urea, *i.e.* the proportion of these substances taken up by the kidneys from the blood passing through them. Urea or creatinine was given intravenously to

rabbits, and at various times afterwards the percentage of the substance in the renal vein blood was compared with the percentage in the arterial blood.

The elimination of creatinine has not been studied in this way by previous workers: that of urea has been studied by Picard [1856], Addis and Shevky [1917], Rhoads, van Slyke, Hiller and Alving [1931], and Dunn, Kay and Sheehan [1931]. Of these, the work of Picard is of only historic interest. That of Addis and Shevky will be considered in detail later. Rhoads, van Slyke, Hiller and Alving utilized an excellent technique, but only their main conclusions are as yet available. They state that the proportion of urea removed from the blood during its passage through the kidney remains constant whatever the height of the blood urea, apart from a few occasions when there is a complete cessation of removal of urea from the renal blood, and the renal vein blood urea rises as high as, and sometimes higher than, the arterial blood urea. It should be noted that these workers used dogs from which one kidney had usually been removed; their findings are in certain respects different from the results obtained with rabbits with two functioning kidneys reported in this paper. The work of Dunn, Kay and Sheehan dealt with the elimination of urea in rabbits with no previous administration of urea.

The excretion of urea in the urine has been studied by many workers: that of creatinine has been the subject of much recent enquiry [Major, 1923; Heesch and Tscherning, 1926; Rehberg, 1926; Cope, 1931; Shannon, Jolliffe and Smith, 1932, etc.].

TABLE I. Urinary excretion of urea and creatinine. Mean figures for five rabbits observed 16 days each.

Rabbit	Wt. kg.	Daily urine			Ratio Urea Creatinine
		Volume c.c.	Urea mg.	Creatinine mg.	
A 1	2.05	71	1870	106	18
A 2	2.27	87	1410	75	19
A 3	2.00	120	1810	84	22
A 4	2.72	118	2320	139	17
A 5	2.04	84	1230	69	18
Mean	2.20	96	1730	95	18

The normal excretion of creatinine by the rabbit has not been previously investigated very thoroughly, and it was therefore considered useful to observe five normal animals for a period of 16 days each; the figures are summarized in Table I.

The daily amounts of urine and of the urea and creatinine were rather variable, but the ratio of urea to creatinine in the urine was much more constant both from animal to animal and from day to day, and averaged 18. The average daily excretion of creatinine was 43 mg. per kg. of body weight. It is not possible to make any accurate calculations from these data as to the relative plasma clearances of creatinine and urea at normal levels in the rabbit, as the usual "blood creatinine" in this animal is only about 1.3 mg. per 100 c.c., and possibly nearly half of this amount may be "chromogenic substance" [see Cope, 1931].

Attempts were made to compare the creatinine contents of arterial blood and renal vein blood in the normal rabbit, but these were unsuccessful as the creatinine contents are so low that accurate colorimetry is impossible. Thus figures cannot be given for comparison with the figures published previously for the renal elimination of urea at normal levels.

The present work deals with the renal elimination of urea and creatinine when these substances have been raised to abnormally high levels in the blood. The investigation thus gives information only about the activities of the kidney under the specific conditions of overload, and deductions from the results as to normal activities of the kidney may be made only with great caution. Nevertheless, these abnormal amounts of normal substances are presumably thrown on the normal renal mechanism, and the experiments may thus be considered to relate to the activity of this normal mechanism under strain. Alternatively, the abnormal amounts of the substances are dealt with by some accessory renal mechanism, which cannot be regarded as utterly abnormal, and whose scope of activity is a problem of importance in renal physiology.

#### THEORETICAL BASIS AND METHODS.

To find the renal extraction ratios two types of operative procedure were employed: the *long sample* and *short sample* experiments. These experiments consisted of injecting the substances into an ear vein, collecting blood from the left renal vein, and making comparisons as follows:

(1) *Long sample experiment:*

Between the total amounts of the substances

(a) carried by the blood to the kidney, and

(b) retained by the kidney,

during the whole period of the experiment.

(2) *Short sample experiment:*

Between the percentages of the substances in corresponding samples of

(a) renal artery blood, and

(b) renal vein blood,

at a specific time after the injection.

The general principles of these experiments have been described previously [Sheehan, 1931]. Certain modifications were necessary to suit the conditions of the present study; these are considered below. It will be seen from the basic principles of the experiments that the long sample experiment requires the injection into the blood stream of the substance to be examined, even if this substance is normally present in the blood. The short sample experiment, on the other hand, can be used to find the renal extraction ratio of any blood constituent either at its normal level or after raising the level artificially by an intravenous injection. For the sake of clearness, creatinine alone is referred to in the following account, but it is to be understood that the theoretical discussion applies also to urea or any other substance which might be used.

#### GENERAL DETAILS.

The animals used were rabbits weighing usually between 2 and 2.5 kg. They were anaesthetized by an intravenous injection of 10 c.c. per kg. of 20 p.c. solution of urethane in physiological saline given at a rate of 2 c.c. per min.; the operation was never begun earlier than 5 min. after the end of the injection of the urethane. Occasionally if an animal was left for over half an hour after giving the urethane, a small second injection of anaesthetic was required.

Careful study was made of the possible effect of variation in the time between giving the urethane and beginning the operation, and this point was further investigated experimentally after the effect of the other variable factors had been established. These controls showed that the variations in the method of giving the anaesthetic did not affect the experimental results at all.

Four experiments have been discarded as the animals showed gross chronic nephritis; in two of them the renal function was decidedly sub-normal.

The renal extraction ratio was always calculated to the nearest whole number; this is about the limit of significance of any results.

## LONG SAMPLE EXPERIMENT.

*Basic principles.*

If creatinine be injected into the blood stream *via* an ear vein, some of it will go in the blood along the renal artery to the kidney during, say, the next 60 sec. Of this, part will be taken up by the kidney parenchyma, part will pass on through the capillaries and leave the kidney by the blood in the renal vein. The amount leaving the kidney can be found by collecting all the blood leaving by the renal vein from the beginning of the injection into the ear vein to the end of the experiment, and determining the total amount of creatinine in this blood. The total amount taken up by the kidney parenchyma can be found by removing the kidney at the moment the collection of the renal vein sample is ended, performing the appropriate determinations on the kidney and applying a correction for the amount of creatinine in the kidney before the experiment. The sum of these two amounts is the amount of creatinine which came to the kidney *via* the renal artery during the experiment.

The renal extraction ratio is the ratio between the amount taken up by the kidney and the amount reaching the kidney during the experiment; expressed as a percentage it is

$$100 \times \frac{\text{Amount taken up by the kidney}}{\text{Amount taken up by the kidney} + \text{amount leaving kidney in renal vein blood}}.$$

The method of determining the correction for the creatinine and urea in the kidney before the experiment is discussed in the appendix. The allowance amounted to 0.4 mg. of creatinine and 8.8 mg. urea per kidney. Its effect is to make the renal extraction ratios a little lower than they would have been without correction; the greatest reduction in the case of creatinine is 3 p.c., and in the case of urea 7 p.c.

The long sample experiment is limited in its application to the first minute after the injection of the creatinine as the amount of blood removed is too large for the collection to be continued longer than a minute. On the other hand it is easier and more reliable than a short sample experiment performed during the first minute, for this latter experiment then requires the introduction into the calculations of a correction, which is large in comparison with the small differences between the creatinine contents of the heart and renal vein blood, and the possible error is thus too big for reliable conclusions to be drawn. Nevertheless, under absolutely standard conditions and when the substances to be examined are suitable, the two types of experiment give the same results, as has been shown in previous work on dyes.



*Technique.*

The technique of the operation is similar to that described by Sheehan [1931], but the renal vein blood and heart blood were collected by the improved method described by Dunn, Kay and Sheehan [1931]. In a number of cases the heart blood was obtained by opening the thorax and needling the left ventricle directly; this is a very efficient and rapid method of collecting heart blood, and the urea or creatinine content of the blood does not appear to be affected during the few seconds required for the collection. The syringes for collecting the blood contained 0.05 c.c. of 20 p.c. potassium oxalate solution; a correction was made in all figures for this volume. The amount of blood collected from the renal vein varied from 8.6 to 23.0 c.c.; the time of collection was usually 1 min. from the beginning of the injection of the creatinine into the ear vein.

In all the creatinine experiments the initial injection of creatinine was given in the standard time of 10 sec. A 10 p.c. solution in physiological saline was used. A 10 p.c. urea solution was used in all urea experiments except the long sample ones, in which a serious difficulty was found. The rapid intravenous injection of strong urea solutions produces a very marked renal vaso-constriction for about half a minute; the slow injection of a large volume of weaker solution produces much less effect. On the other hand, as will be explained in the appendix, it is important in the long sample experiments that the duration of the injection shall not be too long and that the volume of fluid injected shall not be excessively large. The technique which best satisfies the requirements of the experiment without producing severe interference with the renal blood flow was found to be the injection of 25 p.c. urea solution during a period of 30 sec. Nevertheless, the renal circulation rates have varied greatly in the course of each experiment; the rate at the time of the peak of the urea content in the blood cannot therefore be established.

Chemical analyses were performed to determine:

- (a) the total amount of creatinine in the whole volume of blood collected from the left renal vein;
- (b) the total amount of creatinine in the left kidney; and for purposes of control,
- (c) the total amount of creatinine in the right kidney;
- (d) the percentage of creatinine in the heart blood;
- (e) the partition of the blood creatinine between the plasma and corpuscles.

The details of one actual long sample experiment are given below.



vein. In practice, as has been shown in a previous paper [Sheehan, 1931], such an experiment is not possible in the rabbit. Actually it is necessary to collect the renal vein blood sample first, and a sample of arterial blood from the heart as soon as possible afterwards. If the injection is given several minutes before the operation, this heart blood sample has practically the same creatinine content as the heart blood just before the collection of the renal vein blood; if necessary a correction can be made for the small reduction which occurs during the time. The method of ascertaining the requisite correction is discussed in the appendix.

The calculation of the renal extraction ratio is a matter of elementary proportion.

Renal extraction ratio, p.c.

$$= 100 \times \frac{\text{difference between creatinine content of heart blood and renal vein blood}}{\text{creatinine content of heart blood}}.$$

#### *Technique.*

The creatinine, in 10 p.c. solution, was injected from 2 to 160 min. before the operation; in this point the experiments differed from those previously reported with dyes, where the interval was only a few seconds. The operative technique is described by Dunn, Kay and Sheehan [1931].

The urea was also given in 10 p.c. solution in isotonic saline. The dose was usually 1000 mg. per kg., but was varied, in some cases, from 350 to 1820 mg. per kg. An attempt was also made to study the renal extraction ratio at very high blood urea levels, but the large amounts of urea which it was necessary to inject proved to be too toxic to the rabbits. In the whole group of short sample experiments the operation was performed at various times from 3 to 213 min. after the injection of urea. The blood ureas lay between 47 and 296 mg. per 100 c.c. Measurements of the urea in the kidneys were not made in any of these experiments.

Chemical analyses were performed to find:

- (a) the percentage of creatinine in the heart blood;
- (b) the percentage of creatinine in the renal vein blood; and, for purposes of control,
- (c) the total amount of creatinine in the kidneys;
- (d) the partition of the blood creatinine between the plasma and corpuscles.

The details of one actual short sample experiment are given below.

*Typical protocol of short sample experiment.*

R. 563. Female. Wt. 2.15 kg. 17. xii. 31.

*Pre-operative.*

0-10 min. 20 c.c. urethane 20 p.c. solution i.v.  
 63 „ 10 c.c. creatinine 10 p.c. solution i.v.  
 77 „ Short sample experiment (14 min. after creatinine).

*Operation.*

0 sec. Laparotomy commenced.  
 11-18 „ Collection of blood from left renal vein (2.5 c.c. in 7 sec.).

c.c. of blood collected	1	2
Time in sec.	14	16

18 sec. Left kidney removed.  
 20 „ Right kidney removed.  
 19-23 „ Collection of blood from heart.

*Estimations.*

Left kidney	6.5 gm.	162 mg. creatinine	per 100 g.
		10.5 mg. „	total.
Right kidney	6.5 gm.	162 mg. „	per 100 g.
		10.5 mg. „	total.
Heart blood	7.1 c.c.	121 mg. „	per 100 c.c.
Renal vein blood	2.5 c.c.	113 mg. „	„ 100 c.c.
Heart blood plasma		120 mg. „	„ 100 c.c.

Heart blood has 61 p.c. of plasma.

*Calculations.*

$$\text{Renal circulation rate} = \frac{2.5 \times 60}{6.5 \times 7} = 3.3 \text{ c.c. per g. per min.}$$

$$\text{Whole blood renal extraction ratio} = \frac{100 (121 - 113)}{121} = 7 \text{ p.c.}$$

The plasma from 100 c.c. heart blood contains 61 p.c. of 120 mg., i.e. 73 mg. creatinine.

$$\text{Plasma renal extraction ratio} = \frac{100 (121 - 113)}{73} = 11 \text{ p.c.} \quad *$$

## EXPERIMENTAL RESULTS.

## CREATININE.

*Long sample experiments.**The renal extraction ratios.*

Eight satisfactory experiments were performed, the more important details of which are given in Table II.

The renal extraction ratios during the first minute after the injection of the creatinine lie between 39 and 21 p.c., with a mean of 28 p.c. The significance of these renal extraction ratios will be considered later.

TABLE II. Creatinine long sample experiments.

Rabbit	Amount of creatinine injected mg.	Duration of collection of renal vein blood sec.	Amount of creatinine accumulated in left kidney (observed amount minus allowance of 0.4 mg. for pre-existent) mg.	Total amount of creatinine in renal vein blood mg.	Renal extraction ratio p.c.
B 1	500	38	13.1	20.6	39
B 2	500	40	6.4	13.8	32
B 3	500	25	9.7	26.2	27
B 4	200	60	3.3	8.8	27
B 5	200	60	4.2	11.9	26
B 6	500	60	7.2	21.9	25
B 7	500	70	9.0	28.1	24
B 8	500	66	8.1	30.1	21

*The amounts of creatinine in the kidneys.*

Some interest attaches to the rapidity with which the creatinine accumulates in the kidneys during the first minute after injection. Table III gives the percentage of injected creatinine found in the two kidneys together at various times after the injection.

TABLE III. Creatinine long sample experiments.

Rabbit	Mean time of removal of kidneys: sec. after beginning of injection	Amount of injected creatinine accumulated in kidneys (observed amount minus allowance of 0.8 mg. for pre-existent) mg.	Total amount injected mg.	Percentage of total amount injected accumulated in kidneys
B 1	40	25.4	500	5.08
B 2	40	13.4	500	2.68
B 3	26	18.8	500	3.76
B 4	61	6.2	200	3.10
B 5	61	8.9	200	4.45
B 6	63	15.0	500	3.00
B 7	70	18.9	500	3.78
B 8	69	16.0	500	3.20
Mean	54			3.63

The mean percentage of creatinine accumulated in the two kidneys together at about a minute after the injection was 3.63. The mean weight of the animals was 2.53 kg. and of their two kidneys together 17.0 g.; *i.e.* the kidneys were only 0.67 p.c. of the body weight but took up more than five times this percentage of the injected creatinine within a minute or less after the injection. This figure of 3.63 p.c. at 1 min. is in no way surprising. It is in satisfactory agreement with what is known of the rate at which other substances accumulate in the kidney. Thus, for example, during the first minute after injection, phenol red has a renal

extraction ratio about twice that of creatinine, and it does not disappear from the general blood stream quite so quickly [Sheehan, 1931]. It would therefore be expected that the amount of phenol red taken up by the kidneys within a minute after injection would be rather more than twice the amount of creatinine taken up in the same time, assuming normal renal circulation rates in both cases. Actually the mean amount of phenol red taken up is 8.4 p.c. of that injected [Sheehan, 1931], *i.e.* two and a third times the percentage of creatinine which accumulates in the kidneys in the same time.

*The blood flow through the kidneys.*

The renal circulation rates in these experiments were quite normal in range, as will be seen from Table IV. The actual rate of the blood flow from the renal vein was usually very steady; in only two animals did the flow slow down to any recognizable extent towards the end of the collection. In these two cases the collection was stopped at once.

Any such reduction of the renal circulation rate towards the end of the collection is probably due to generalized vasomotor disturbance. The blood flow to the left kidney does not appear to have been disturbed by the operative interference with its vein. This is shown by the close agreement between the amounts of creatinine which accumulated in the two kidneys from any animal. The relative amounts of creatinine in the two kidneys may be taken as a fairly good indication of the relative

TABLE IV. Creatinine long sample experiments.

Rabbit	Renal circulation rate c.c. per g. per min.	Total kidney creatinine left/right
		p.c.
B 1	1.7	106
B 2	1.7	92
B 3	3.4	106
B 4	2.0	112
B 5	1.9	90
B 6	2.0	93
B 7	2.1	91
B 8	2.4	103
Mean	2.15	99

blood flows through these kidneys, since the amount of creatinine in the blood is presumably the same for both sides. The amount of creatinine in the left kidney expressed as a percentage of that in the right kidney is shown in Table IV.

*Short sample experiments.**The renal extraction ratios.*

The results of the short sample experiments are collected in Table V, arranged according to the time elapsing between the injection of creatinine and the operation.

It will be seen that the renal extraction ratios lie between 6 and 23 p.c.

TABLE V. Creatinine short sample experiments.

Rabbit	Creatinine injected		Renal circulation rate c.c. per g. per min.	Creatinine mg. per 100 c.c. blood		Renal extraction ratio p.c.
	mg. per kg.	min. before operation		Heart	Renal vein	
C 1	225	2	1.9	67	56	16
C 2	470	3	1.4	160	145	9
C 3	215	4	2.6	59	54	8
C 4	500	5	2.3	114	100	12
C 5	230	6	1.3	64	53	17
C 6	490	8	1.5	169	159	6
C 7	210	12	2.5	41	32	22
C 8	465	14	3.3	121	113	7
C 9	200	19	3.3	31	29	6
C 10	225	26	2.8	32	27	15
C 11	500	26	3.5	99	85	14
C 12	500	44	1.7	66	56	15
C 13	500	60	2.6	64	56	13
C 14	500	90	1.9	32	25	22
C 15	500	100	0.9	46	37	20
C 16	500	105	2.8	22	17	23
C 17	2000	112	0.8	154	146	6
C 18	1600	121	0.8	188	170	10

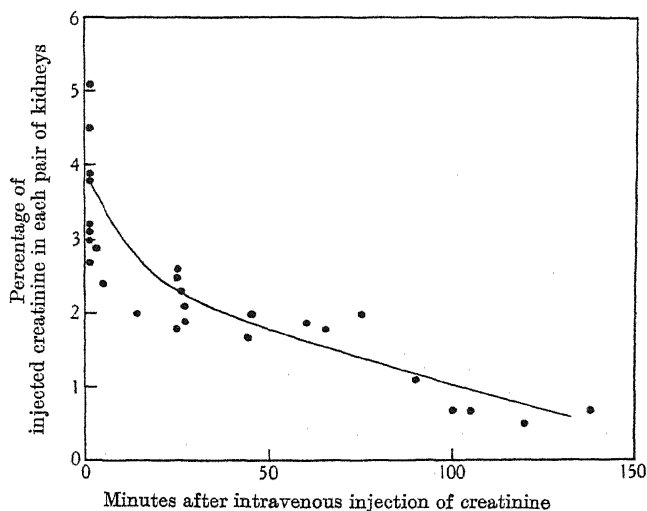


Fig. 1. Amounts of creatinine in kidneys.

*The amounts of creatinine in the kidneys.*

The amounts of creatinine found in the kidneys in the short sample experiments are of interest in relation to the figures from the long sample experiments. In Fig. 1 the total for each pair of kidneys is given as a percentage of the total amount injected, 0.4 mg. per kidney for pre-existent creatinine being deducted before calculating the percentages. The figures from the long sample experiments and from a few control experiments are also plotted.

It will be seen that the amount of creatinine in the kidneys is at its maximum immediately after the injection of the creatinine and that it then steadily diminishes.

*Additional experimental results.*

For the purpose of the later discussion of the significance of renal extraction ratios, it is necessary to know what proportion of the injected creatinine is carried in the plasma as compared with that carried in whole blood. This requires consideration of two factors, the relative volumes and the creatinine contents of plasma and corpuscles.

*Distribution of creatinine in the blood.*

Investigations were made as to the distribution of creatinine between plasma and corpuscles at various times after the intravenous injection.

TABLE VI. Distribution of creatinine between plasma and corpuscles.

Time after injection of creatinine min.	Creatinine mg. per 100 c.c.		Volume of plasma per 100 c.c. blood c.c.	Ratio of amount of creatinine in plasma of 100 c.c. blood to that in 100 c.c. blood p.c.
	Whole blood	Plasma		
1	31	43	70	97
1	122	150	65	80
1	76	95	63	79
1	142	169	65	77
1	58	63	61	67
2	56	59	61	73
2	62	75	54	66
4	52	52	56	56
13	35	32	69	63
13	30	30	55	57
14	117	120	—	—
23	30	27	53	47
25	89	82	60	55
25	54	51	56	54
25	29	26	59	52
26	15	16	57	60
27	29	29	65	65
27	11	11	55	55
101	100	99	62	62
107	67	66	60	58



About 10 c.c. of blood were used. The requisite amount of this blood was taken for analysis, and the rest was spun, at once, on a rapid centrifuge for 3 min.; this yielded more than the 2 c.c. of plasma required for the estimation of its creatinine content. The remainder of the blood was again centrifuged for determinations of corpuscular volume. The results are shown in Table VI. The table may be summarized by certain averages as follows:

Time after injection of creatinine min.	Ratio of amount of creatinine in 100 c.c. plasma to that in 100 c.c. blood p.c.	Ratio of amount of creatinine in plasma of 100 c.c. blood to that in 100 c.c. blood p.c.
1	123	80
3	109	65
25	97	55
105	98	60

Two points need mention. First, it is not possible to say how much diffusion of creatinine occurs between the plasma and corpuscles during the manipulations *in vitro*, such as pipetting and centrifugation. The figures do not therefore give absolutely reliable information about the conditions *in vivo*. As, however, the manipulations *in vitro* were the same for all the bloods, there is clearly some significance in the change of general level of the figures with the lapse of time after the injection. Second, the blood used for the determinations at 1 min. was that from the renal vein. The plasma of this blood had been exposed to the action of the kidney and therefore had lost part of its creatinine; probably it had a rather higher creatinine content, relative to that in whole blood, when it was in the renal artery.

The only legitimate conclusions are as follows. Injected creatinine, which is only in the plasma at the time of the injection, passes gradually into the corpuscles. An equilibrium is established in about 5 or 10 min. with a fairly equal concentration of creatinine in plasma and corpuscles. As the plasma creatinine is steadily reduced by the activity of various organs the concentration in the corpuscles falls in association; but there appears to be some lag in the return diffusion, and the concentration in the corpuscles may thus remain for a time slightly higher than that in the plasma. The absolute amount of creatinine in the blood is eventually distributed between plasma and corpuscles roughly in proportion to their relative volumes; *i.e.* about 60 to 40.

#### *Plasma volumes.*

It is convenient at this stage to give the data on which the relative volumes of plasma and corpuscles have been established. In a discussion

in a previous paper [Dunn, Kay and Sheehan, 1931] the corpuscles were assumed to be half the blood volume; the proportion of plasma required to be filtered in the glomeruli to account for the elimination of urea by filtration was therefore taken to be twice the renal extraction ratio. This appears to be erroneous; the present evidence shows that the factor should have been 1.6 instead of 2.

The figures in Table VII were obtained by the thorough centrifugation of about 10 c.c. of blood from each rabbit; the time required for the corpuscles to reach a constant volume was 30-50 min. The blood contained a known amount of 20 p.c. solution of potassium oxalate, in some cases as much as 0.2 c.c., which is sufficient to make the corpuscles shrink to about one-twentieth of their volume [Osgood, 1926]. A correction has been made in each case for the contraction of the corpuscles and also for the volume of oxalate solution added.

Table VII shows the percentage of plasma in:

- (a) ear vein blood of normal rabbits with no previous injections;
- (b) ear vein blood of rabbits injected with creatinine solution several minutes previously;
- (c) heart blood of rabbits injected with urethane solution several minutes previously;
- (d) heart blood of rabbits injected both with urethane and creatinine solution several minutes previously, and then subjected to short sample experiments;
- (e) renal vein blood of rabbits injected with urethane solution several minutes previously, and then subjected to long sample experiments involving the injection of creatinine solution intravenously.

TABLE VII. Volume of plasma as percentage of blood volume in individual rabbits.

Normal (a)	Intravenous injection of		Intravenous injection of creatinine and urethane	
	Creatinine (b)	Urethane (c)	Short sample exp. (d)	Long sample exp. (e)
59	50	60	55	61
61	54	61	56	62
62	55	63	57	63
65	56	63	59	63
65	59	63	59	64
74	61	66	59	65
	69	67	60	65
	69		65	66
			66	70
Mean 64.3	59.1	63.3	59.6	64.3

It will be seen that there are very wide variations between different rabbits, but that the average figure is about 62 p.c. The small differences between the means cannot be considered of any particular significance. Even the long sample experiments, where 2 or 5 c.c. of creatinine solution were injected at the beginning of the collection of blood, do not show any definite evidence of dilution of the blood as compared with normals, while the figures of the other groups show a very slightly lower percentage of plasma than the normal.

To investigate fine differences it would clearly be necessary to examine a number of samples of blood from the same rabbit. It appears from investigation of three rabbits which were each bled from the ear on two separate occasions, with a fortnight's interval for examination of the relative amounts of the blood constituents, that the plasma volume is a fairly constant percentage of the blood volume in each individual animal.

The main conclusion to be drawn from these results is that, although the relative volume of the plasma is different in different rabbits, the intravenous injection of urethane and creatinine in the way described in this paper has no recognizable influence on it. This rapid and efficient adjustment of the blood volume is similar to that noted by Smith and Mendel [1920] who, in 2 min., injected 100 c.c. of isotonic solutions of salts intravenously in rabbits and produced only a very transient hydræmia; the blood volume was only raised about 15 p.c. 10 min. later.

## UREA.

### *Long sample experiments.*

#### *The renal extraction ratios.*

Five experiments are recorded, the details of which are summarized in Table VIII. 25 p.c. urea solution was injected.

TABLE VIII. Urea long sample experiments.

Rabbit	Amount of urea injected g.	Duration of collection of renal vein blood sec.	Amount of urea accumulated in left kidney (observed amount minus allowance of 8.8 mg. for pre-existent) mg.	Total amount of urea in renal vein blood mg.	Renal extraction ratio p.c.
D 1	4.85	40	86	125	41
D 2	5.64	60	57	84	40
D 3	4.88	60	113	275	29
D 4	2.69	60	22	60	26
D 5	3.63	60	24	85	22
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The renal extraction ratios lie between 41 and 22 p.c., with a mean of 32 p.c. The significance of these figures will be dealt with later.

*The amounts of urea in the kidneys.*

The rate of accumulation of urea in the kidneys during the first minute after injection is shown in Table IX.

TABLE IX. Urea long sample experiments.

Rabbit	Mean time of removal of kidneys: sec. after beginning of injection	Amount of injected urea accumulated in kidneys (observed amount minus 17.6 mg. for pre-existent) mg.	Total amount injected mg.	Percentage of total amount injected accumulated in kidneys
D 1	42	183	4850	3.77
D 2	61	143	5640	2.53
D 3	62	223	4880	4.57
D 4	61	45	2690	1.67
D 5	61	46	3630	1.27
Mean	57			2.76

The mean, 2.76 p.c., is decidedly lower than that of creatinine, which was 3.63 p.c. It is nevertheless an indication of rapid accumulation. The average weight of the kidneys was 14.5 g. and that of the animals was 2.26 kg.; the kidneys were therefore 0.64 p.c. of the body weight, but took up over four times this percentage of the injected urea.

The lower proportion of urea taken up as compared with creatinine is probably related to the poorer renal circulation in the urea series at the time of the peak of the blood content. Certainly the renal extraction ratios are slightly higher than in the corresponding creatinine experiments, and it would therefore seem that the kidney is able actually to take up more urea than creatinine if equal amounts of the two substances reach it in the blood.

*The blood flow through the kidneys.*

The renal circulation rates in these experiments shown in Table X can at best be only a poor indication of the fluctuating blood flows. It is nevertheless rather surprising to find them as high as they are; the blood flow before and after the period of marked vaso-constriction must have been relatively large. Although the blood flow varied so much, it appears to have been the same on both sides. This is shown by the fact that the amounts of urea accumulated in the two kidneys lie within the range

of  $\pm 10$  p.c. in each animal and agree satisfactorily on the whole for all animals. D 2 is excluded from this statement; it appears probable that the blood flow was less through its left kidney than through its right.

TABLE X. Urea long sample experiments.

Rabbit	Renal circulation rate c.c. per g. per min.	Total urea in kidneys left/right p.c.
D 1	2.6	90
D 2	(1.2)	(70)
D 3	2.6	103
D 4	1.7	94
D 5	1.6	103
Mean (excluding D 2)	2.11	98

*Short sample experiments.*

*The renal extraction ratios.*

The results of the short sample experiments are shown in Table XI, arranged according to the time elapsing between the injection of the urea and the operation. It will be seen that the renal extraction ratios lie between  $-5$  and  $8$  p.c. A negative extraction ratio means that the renal vein blood contains more urea than the heart blood.

TABLE XI. Urea short sample experiments.

Rabbit	Urea injected		Renal circulation rate c.c. per g. per min.	Urea mg. per 100 c.c. blood		Renal extraction ratio p.c.
	mg. per kg.	min. before operation		Heart	Renal vein	
E 1	1000	3	0.9	205.0	203.2	1
E 2	1000	3	1.5	206.1	198.7	4
E 3	1000	8	3.0	154.9	144.6	7
E 4	1000	8	3.4	166.2	163.0	2
E 5	540	16	0.9	92.1	96.7	-5
E 6	1000	17	1.9	167.0	164.8	1
E 7	400	17	1.2	47.2	47.8	-1
E 8	1000	18	0.8	171.5	174.0	-1
E 9	950	26	0.8	155.8	163.2	-5
E 10	350	33	1.7	103.7	99.5	4
E 11	1000	35	1.1	160.7	157.0	2
E 12	1050	39	1.3	188.5	188.3	0
E 13	650	45	5.6	116.1	114.7	1
E 14	1710	45	1.3	232.0	277.3	2
E 15	430	46	3.1	80.5	78.3	3
E 16	1820	55	2.8	197.5	188.4	5
E 17	770	70	3.0	137.5	130.0	5
E 18	1000	105	2.3	111.5	106.7	4
E 19	1000	118	3.8	121.1	114.0	6
E 20	1000	147	3.8	132.2	125.1	5
E 21	1000	159	1.9	118.4	108.5	8
E 22	4000	174	4.1	296.2	281.7	5
E 23	1000	189	0.9	80.1	73.5	8
E 24	1000	212	1.2	82.2	77.0	6
E 25	1000	213	3.8	101.2	93.1	8

The figure given for the blood urea content of heart or renal vein blood is the mean of two paired determinations on each sample, agreement between which was usually within 0.3 mg. per 100 c.c.

*The blood flow through the kidneys.*

In all the short sample experiments the renal circulation rates show the usual wide variations; the average figure is 2.2 c.c. per g. per min. which is about the normal. There is, however, a tendency to low rates for about half an hour after the injection of the urea, and to high rates later. This can be shown by the averages from the short sample experiments at various times after the injection.

Time after injection of urea min.	Number of rabbits	Mean renal circulation rate c.c. per g. per min.
0-25	8	1.7
25-50	7	2.1
50-220	10	2.8

This suggests that the renal vaso-constriction which develops at the time of the injection continues to some extent for several minutes and then gives place to a renal vaso-dilatation. An average renal circulation rate of 2.8 c.c. per g. per min. in any series of ten animals is unusually high and must be regarded as significant. It is of interest to note that Addis [1928] has shown that the kidney function of an animal is at its highest when large amounts of urea and water have been given by mouth some time previously. The renal blood flow appears to be stimulated by this treatment in the same way as it is increased by the intravenous injection of urea about an hour before.

The variations of the renal circulation rates corresponding to the time after the injection of urea are superimposed on the variations which occur in normal animals. This association between the variations is of course not a methodical one; the frequency of occurrence of any particular rate in these experiments is therefore not significantly different from that in normal rabbits. This is seen from the collected results of all the animals from the urea and creatinine experiments, including those discarded on account of poor blood flows or for other reasons. They are shown in Table XII, together with the results of a few similar experiments involving the injection of sodium sulphate. The percentages of animals whose renal circulation rates lay within the individual 1 c.c. ranges detailed below are very close in the present series of 113 measurements to the percentages in a series of 120 experiments which have been recorded previously [Sheehan, 1932*a*]. The mean rate for the sulphate

experiments is rather high; this is in accord with the calculations made by Ekehorn [1931] from the data of Mayrs and Watt as to renal blood flows in rabbits injected with sulphate.

TABLE XII. Renal circulation rates.

Renal circulation rate c.c. per g. per min.	Percentage of measurements in each 1 c.c. range	
	Present series 113 rabbits	Previous series 120 rabbits
0-1	21	14
1-2	35	41
2-3	22	29
3-4	18	12
4-5	3	3
5-6	1	1
Mean renal circulation rates:		
Previous series:	2.0 c.c. per g. per min.	
Present series:		
All rabbits	2.1	„ „
53 rabbits injected with urea	2.1	„ „
41 „ „ creatinine	1.9	„ „
19 „ „ sulphate	2.7	„ „

## THE RELATIONSHIPS OF THE RENAL EXTRACTION RATIOS.

### CREATININE.

#### *Long sample experiments.*

The renal extraction ratios lie between 21 and 39 p.c. The range is rather wide and needs some explanation. As in the short sample experiments to be discussed below, the various factors which could possibly influence the ratios have been carefully studied. The only relationships which can be established are:

(1) The highest three renal extraction ratios are those from the shortest three experiments.

(2) There is a tendency to an inverse proportionality between the renal extraction ratio and the renal circulation rate similar to that noticed in the case of dyes during the first minute after injection. This tendency is partly obscured by B 3 which has a high renal circulation rate, but a short time of collection of renal vein blood.

The combined influence of the time of collection and the circulation rate factors is expressed in the volumes of renal vein blood collected. These, as is shown in Table XIII, have an inverse relationship to the renal extraction ratios.

TABLE XIII. Creatinine long sample experiments.

Rabbit	Amount of blood collected from renal vein c.c.	Renal extraction ratio p.c.
B 1	9.9	39
B 2	11.7	32
B 3	13.3	27
B 4	15.2	27
B 5	15.7	26
B 6	15.2	25
B 7	23.0	24
B 8	21.2	21

No definite relationship can be made out between the renal extraction ratio and the actual amount of creatinine coming to the kidney during the experiment. This amount might perhaps be expected to depend on the amount of the blood flow, but such a view is contradicted by the results of the experiments; it does not take into account the differences in the amounts of creatinine injected and the different heights of the peaks of blood creatinine even after the injection of the same amounts of creatinine.

*Short sample experiments.*

The renal extraction ratios for the short sample experiments are definitely lower than those for the long sample experiments. The fairly wide range of variation which they show among themselves again indicates the need for considering what influence the numerous variable factors in the experiments have on the ratios.

By graphing all the known variable experimental factors against the renal extraction ratios, two were established as significant, and will later be discussed fully. With these in mind, the others were re-examined, but with negative results, and are here enumerated without elaboration.

- (1) Weight, sex, fatness, calculated blood volume of animal.
- (2) Amount and dosage of creatinine injected.
- (3) Volumes of solutions of creatinine and urethane injected.
- (4) Dose of urethane; time between its injection and operation; depth of anaesthesia. (These were fairly constant.)
- (5) Amount of operative interference. (Fairly constant. Where the collection of renal vein blood was unduly prolonged, the experiments were discarded.)
- (6) Renal circulation rates. The suggestion of a direct relationship between these and the extraction ratios, especially at high and low values, is not conclusively established by these experiments.



The two significant factors are to some extent inter-related. They are:

(a) the time elapsing between the injection of the creatinine and the operation;

(b) the amount of creatinine in the heart blood.

On graphing the renal extraction ratios against the time after the injection a very definite relationship can be recognized. The ratios during the first minute, as ascertained by the long sample experiments, are usually about 27 p.c. After this, during the first 20 min., there is a marked tendency to low ratios. From 25 min. onwards the ratios rise

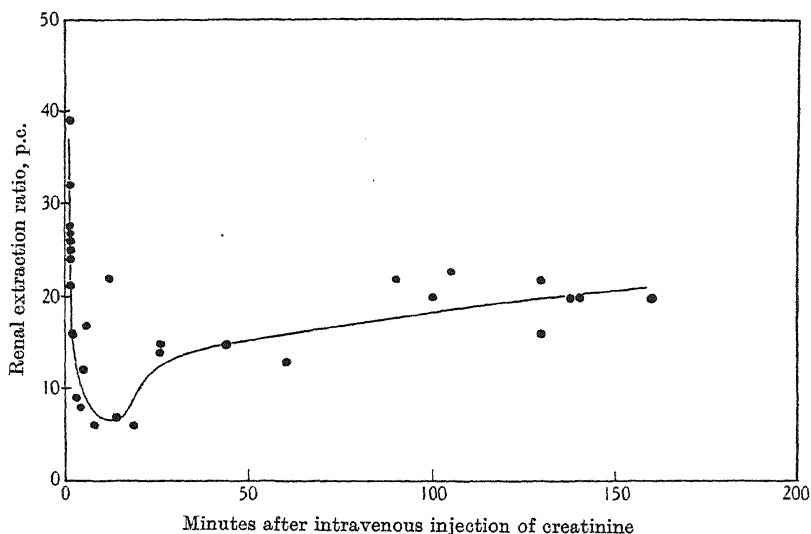


Fig. 2. Relationship of renal extraction ratios for creatinine to time after injection.

steadily to about 20 p.c. This is shown in Fig. 2. Exps. C 17 and C 18 are not included in this Figure as they are essentially examples of the action of the second factor. The experiments of series F are, however, comparable, and are included.

The extraction ratios during the first minute have been discussed earlier. The question that now arises is why there is the dip in the curve at 6-24 min. after the injection. Is this phenomenon related to the time interval alone, or is it the result of some other concomitant factor?

The key to the problem lies in the figures after 24 min. These ratios are from a time when the interfering factor of the dip in the curve has passed off.

These experiments, C 10-C 18, are graphed in Fig. 3 against the height of the blood creatinine. The figures of ten further short sample experiments of series F and G are also included; these are experiments about 2 hours after the administration of the creatinine, and the "time dip" is thus not a complicating factor. It is clear from the Figure that the renal extraction ratios tend to an inverse relationship with the height of the blood creatinine.

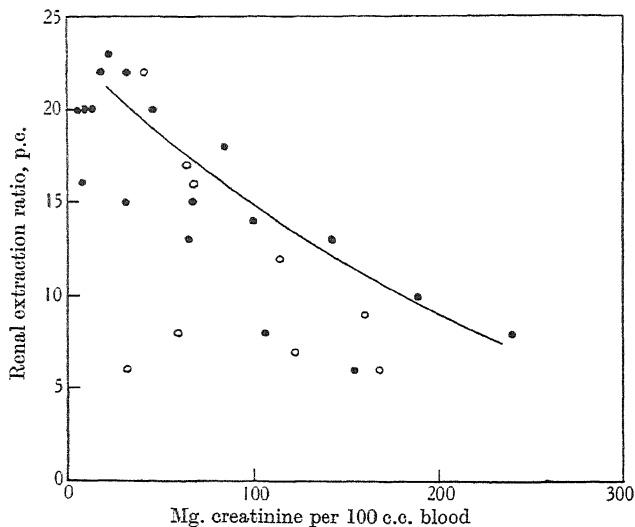


Fig. 3. Relationship of extraction ratios for creatinine to height of blood creatinine. ● Experiments later than 24 min. after injection. ○ Experiments earlier than 24 min. after injection.

The Figure also shows by a different notation the renal extraction ratios of C 1-C 9, *i.e.* less than 24 min. after injection. It will be seen that in five cases the low ratios at this time are satisfactorily explained by the height of the blood creatinine; of the other four ratios, two are low and two are unusually low. The results of the urea experiments are of assistance in suggesting an explanation for these four low ratios during the first 24 min. after injection.

The relatively lower efficiency of the kidney in dealing with high blood creatinines than with small ones is of decided interest in relation to Marshall's recent work on the plasma clearance of phenol red in dogs [1931]. This author showed that the kidney is relatively rather less efficient in secreting this dye from high blood concentrations than from low ones.

The point may also be demonstrated by comparing the actual differences between the creatinine content of the heart blood and renal vein blood at different levels of the blood creatinine. Fig. 4 shows that these "differences" increase with the height of the blood creatinine, but at a diminishing rate. The gradual diminution of the renal extraction ratios shown in Fig. 3 is a measure of the failure of the kidney to maintain a strictly proportional increase in the "differences" shown in Fig. 4. From the graph-points for blood creatinines of 20-40 mg. per 100 c.c.

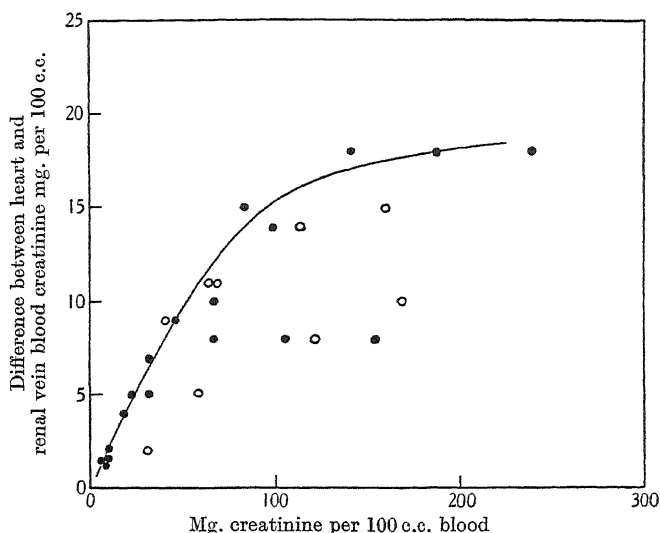


Fig. 4. Relationship of difference between heart and renal vein blood creatinine to height of blood creatinine. ● Experiments later than 24 min. after injection. ○ Experiments earlier than 24 min. after injection.

it might be expected that, if the proportion remained constant, the "difference" would be 25 mg. per 100 c.c. at a blood creatinine of 120 mg. per 100 c.c. Actually the figure is about 17 mg. per 100 c.c. It appears doubtful whether the "difference" in these short sample experiments would ever reach 25 mg. per 100 c.c., no matter how high the blood creatinine were raised. This, however, is only a matter of speculation; the blood creatinine has not been higher than 250 mg. per 100 c.c. in any of the short sample experiments.

It will also be seen from the Figure that the four experiments before 24 min., where the renal extraction ratios were low, have also an unusually low "difference."

This part of the discussion may be summarized in two conclusions:

(1) The difference between the creatinine content of heart blood and renal vein blood increases as the creatinine content of the heart blood rises, but in a steadily diminishing proportion. As a result, there is an inverse relationship between the renal extraction ratio and the height of the blood creatinine.

(2) From 6 to 24 min. after the injection of the creatinine the renal extraction ratio is sometimes lower than would be expected from the relationship of renal extraction ratios to blood creatines at other times.

### UREA.

#### *Long sample experiments.*

The renal extraction ratios lie between 22 and 41 p.c. All attempts to establish a correlation between the renal extraction ratio and the factors which are known to influence it in work with other substances have, however, been unsuccessful. This statement refers essentially of course to the factor of renal circulation rates or actual amounts of blood flow from the kidney. The reason is not far to seek: the blood flows were so variable at the crucial time of the experiment, *i.e.* when the peak of the urea content of the blood was passing through the kidney. The renal circulation rate during that time can certainly not be judged from the blood flow averaged over the whole minute of the experiment; it is therefore not surprising that the renal extraction ratios cannot be related to this averaged blood flow.

One point may, however, be noted. The experiment with the highest renal extraction ratio is that in which the renal vein blood was collected for the shortest time. The same phenomenon was observed in the long sample experiments with creatinine.

#### *Short sample experiments.*

The experiments of series F are included in this discussion as they show no differences from those of series E. The renal extraction ratios for these raised blood ureas are very variable; they lie between -5 and +8 p.c.

As with creatinine, they have been graphed against all observed factors; again the two important factors are the time elapsing between the injection of the urea and the operation, and the amount of urea in the heart blood. The time relationship is considered first; it is shown in Fig. 5.

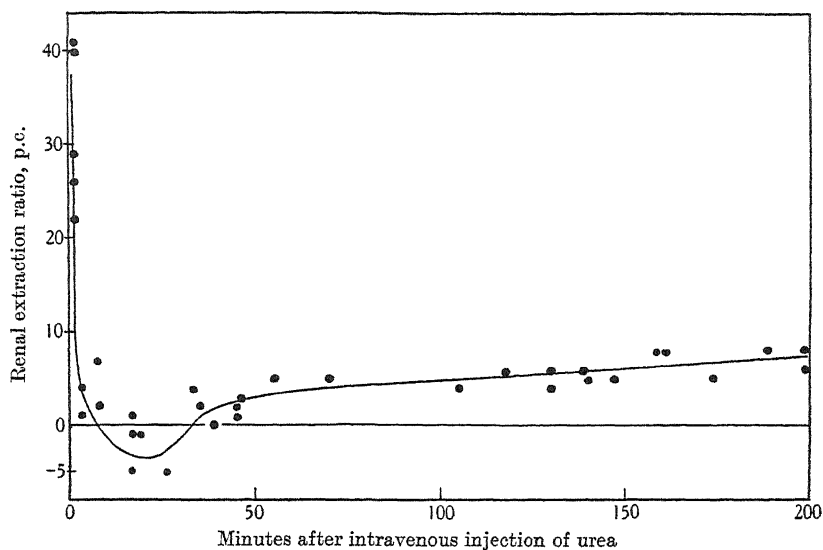


Fig. 5. Relationship of renal extraction ratios for urea to time after injection.

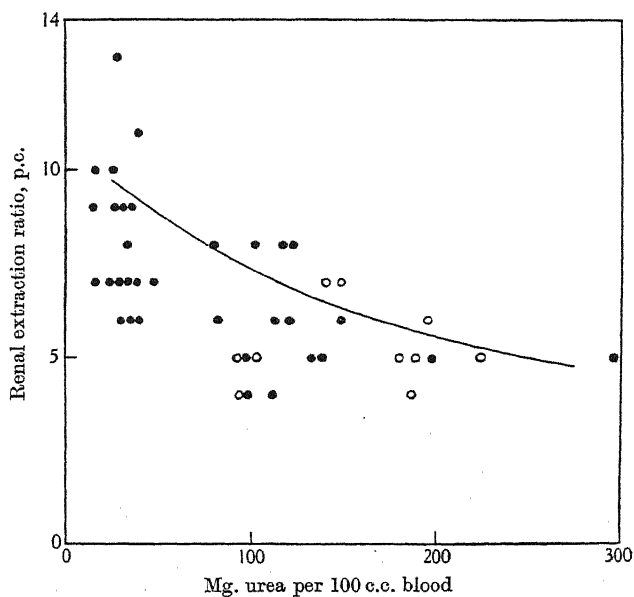


Fig. 6. Relationship of renal extraction ratios for urea to height of blood urea.

● Authors' figures. ○ Figures of Addis and Shevsky.

The Figure shows that there is a dip in the curve during the first 50 min., which is longer and deeper than in the case of creatinine. Between 16 and 26 min. the renal extraction ratios are usually negative, and are sometimes as low as  $-5$  p.c. From 40 to 50 min. the ratios increase rather quickly, and after 50 min. settle to a comparatively constant level, though there is still evidence of a tendency to increase.

The interpretation of the curve rests again on the figures after the preliminary dip, *i.e.* on the figures later than 50 min. after the injection. These fifteen renal extraction ratios appear to be relatively free from the influence of the time factor; they can therefore be graphed against the height of the blood urea. This is done in Fig. 6, which also includes two other groups of figures for comparison. These are, first, a previously published group of eighteen renal extraction ratios for urea on animals with no previous injection of urea [Dunn, Kay and Sheehan, 1931]. These data are quite comparable with those of the present work as the experimental technique was the same. The second group is of ten rabbits with raised blood ureas from the figures of Addis and Shevky [1917]; this group is shown in the Figure by a distinctive sign.

Addis and Shevky employed a technique roughly similar to the short sample experiment. They publish, in all, figures of nineteen experiments where gross bilateral interference with the kidneys was avoided, but give no details of the variable factors such as time, etc. Some division of these figures into groups is necessary, but, as their data are so incomplete, the division can only be attempted here on the general principles which have been established in the present study. This classification of their figures is given below and the figures are given in Table XIV; the fourth group ( $\delta$ ) contains the figures which are used for comparison with those of the present work.

Group  $\alpha$ . At normal blood urea levels, probably from normal animals with no previous or recent administration of urea. The ratios are nevertheless unusually low.

Group  $\beta$ . Low ratios, probably soon after the administration of urea, or in rabbits with low renal function perhaps associated with low renal circulation rates.

Group  $\gamma$ . High figures open to doubt in view of the levels of the extraction ratios in the rest of their work, in the work of Dunn, Kay and Sheehan [1931], and in the short sample experiments of the present work; possibly due to some experimental error.

Group  $\delta$ . Raised blood ureas and satisfactorily functioning kidneys, probably some time after administration of urea.

TABLE XIV. Results of Addis and Shveky's experiments.

Group	Urea mg. per 100 c.c. blood		Renal extraction ratio p.c.
	Jugular vein	Renal vein	
$\alpha$	23	22	5
	27	27	0
	38	38	0
$\beta$	78	77	1
	129	130	-1
	197	196	0
	216	211	2
$\gamma$	53	42	21
	57	47	18
$\delta$	93	88	5
	96	92	4
	102	97	5
	140	130	7
	149	139	7
	178	169	5
	187	180	4
	189	180	5
	196	184	6
	223	212	5

To return to Fig. 6. There is a reasonable agreement of the present results with the group  $\delta$  figures of Addis and Shevsky. It will be seen that the renal extraction ratio is in general lower as the blood urea increases. The inverse relationship between the efficiency of the kidney and the height of the blood urea is a similar phenomenon to that observed in the case of creatinine. It can also be demonstrated by plotting the actual differences between the urea content of renal vein blood and heart blood against the height of the blood urea as in Fig. 7.

The same two groups of figures are again included for comparison; the high blood urea figures of Addis and Shevsky and the normal blood urea figures of Dunn, Kay and Sheehan. The gradual failure of the kidney to keep the "difference" proportional to the blood urea is seen quite clearly. It seems possible from extrapolation that the maximum "difference" that could be reached would be between 15 and 20 mg. per 100 c.c. at a blood urea of about 500 mg. per 100 c.c. This is of course quite hypothetical; as mentioned earlier a number of experiments were planned to deal with this point, but were unsuccessful owing to the toxic effect of the large doses of urea required.

With regard to the group of experiments performed earlier than 50 min. after the injection of the urea, consideration must be given to the question of whether the low renal extraction ratios are indicative merely of the height of the blood urea at the time. By comparison of the ratios

of E 1-E 15 (from the "dip" in the time graph) with the ratios plotted in Fig. 6 it will be seen that E 2 and E 3 gave renal extraction ratios which were normal at their levels of blood urea, that E 10 gave a ratio a little lower than the normal range, and that all the remainder are very definitely subnormal. It is quite clear that the amount of time elapsing after the injection is a factor of great importance during the first 50 min., and that the height of the blood urea is not of the same significance at that time.

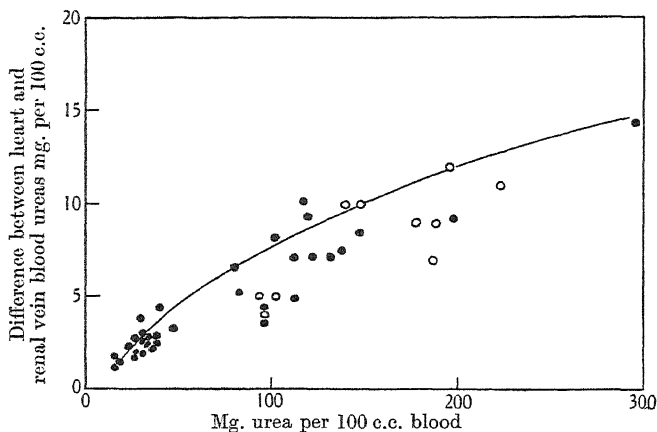


Fig. 7. Relationship of difference between heart and renal vein blood ureas to height of blood urea. ● Authors' figures. ○ Figures of Addis and Shevky.

The conclusions to be drawn from the short sample experiments about urea are almost identical with those already drawn about creatinine.

(1) The difference between the urea content of heart blood and renal vein blood increases as the urea content of the heart blood rises, but in a steadily diminishing proportion. As a result, there is an inverse relationship between the renal extraction ratio and the height of the blood urea.

(2) From 6 to 50 min. after the injection of the urea the renal extraction ratio is almost invariably lower than would be expected from the relationship of renal extraction ratios to blood ureas at other times.

#### COMBINED UREA AND CREATININE EXPERIMENTS.

Having ascertained the two important factors which affect the renal extraction ratios in the cases of creatinine and urea, it is now possible to investigate the ratios for both substances when they are injected together in the same animal. Such an experiment should be performed at an interval after the injection of the urea and creatinine sufficient



to avoid the complicating factor of the "dip" period. The only known factor remaining is the amount of the substance in the blood, and the influence of this factor can be estimated sufficiently closely from Figs. 3 and 6. It is thus possible to find whether the administration of either creatinine or urea interferes with the renal extraction ratio for the other substance.

The purpose of the first series of combined experiments was to raise the blood urea fairly high, but to raise the blood creatinine only sufficiently to allow of accurate estimation: then, by short sample experiment, to find the renal extraction ratios for the two substances, and to compare the ratios with those obtained when each substance is injected singly.

A mixed solution of urea and creatinine was injected intravenously in each rabbit; the doses were always 1000 mg. of urea and 250 mg. of creatinine per kg. The operations were performed at 130–160 min. later. The renal circulation rates were normal, varying between 1.6 and 3.1 c.c. per g. per min. with an average of 2.3 c.c. per g. per min. The blood ureas were about 100 mg. per 100 c.c., the blood creatinines about 10 mg. per 100 c.c. At these levels it will be seen from Figs. 3 and 6 that the renal extraction ratios are about 6 p.c. for urea and 20 p.c. for creatinine when the substances are given singly.

The chief results of the experiments are given as series F in Table XV. It will be seen that the renal extraction ratios lie within the range found when the substances are injected singly. The conclusions from this series of experiments are that a fairly large elevation of the blood urea does not affect the renal extraction ratio for creatinine, and that a small elevation of the blood creatinine does not affect the renal extraction ratio for urea.

A second series of experiments was then performed to test the effect of raising the blood creatinine to high levels while leaving the blood urea

TABLE XV. Combined experiments.

Rabbit	Urea mg. per 100 c.c. blood		Creatinine mg. per 100 c.c. blood		Renal extraction ratio p.c.	
	Heart	Renal vein	Heart	Renal vein	Urea	Creatinine
Large doses of urea:						
F 1	147.6	139.2	18.0	14.0	6	22
F 2	95.7	92.1	8.2	6.9	4	16
F 3	111.2	104.1	9.3	7.5	6	20
F 4	95.9	91.5	7.1	5.7	5	20
F 5	119.9	110.6	10.4	8.3	8	20
Large doses of creatinine:						
G 1	38.1	37.9	240	222	0	8
G 2	50.1	48.9	141	123	2	13
G 3	48.4	48.4	105	97	0	8
G 4	24.9	24.3	84	69	2	18

low. These rabbits received creatinine intravenously in doses from 1.6 to 2.0 g. per kg.; they were given no urea. The short sample experiments were performed at 112–131 min. after injection. The renal circulation rates were low, ranging from 0.8 to 1.2 c.c. per g. per min., with an average of 0.9 c.c. per g. per min. The blood creatinines were raised to 84–240 mg. per 100 c.c. From Fig. 3 it will be seen that at these levels the usual renal extraction ratios for creatinine are about 16 and 8 p.c. respectively.

The results of the experiments are given as series G in Table XV. The renal extraction ratios for creatinine are within the range found in experiments where creatinine was investigated alone. The renal extraction ratios for urea are, however, somewhat surprising. The blood ureas were normal and the renal extraction ratios would therefore be expected to lie between 6 and 13 p.c. [Dunn, Kay and Sheehan, 1931]. They lie in the present experiments at 0 and 2 p.c. instead. There is clearly a marked impairment of urea elimination.

The results of the two series of experiments may be illustrated in another way. The average differences between heart blood and renal vein blood are as follows:

	Difference Heart blood—renal vein blood mg. per 100 c.c.	
	Urea	Creatinine
Series F	6.6	2.3
Series G	0.5	15.0
Normal rabbits	2.5	0.15*

\* Estimated from creatinine excretion in urine.

In series F, in which large amounts of urea were injected, the kidneys were removing absolutely much more urea and creatinine from each 100 c.c. of blood than in the normal rabbit, though the renal extraction ratio for urea was of course lower than in rabbits with normal blood urea.

In series G, in which large amounts of creatinine were injected, there was not only a relative reduction of urea elimination, as expressed in the renal extraction ratios, but an absolute reduction as well. The kidneys were removing only about a fifth of the urea that is removed from each 100 c.c. of blood in normal rabbits. This specific interference with urea elimination is of interest. There was also an interference with the renal circulation, the blood flow being only about half the normal. (See also the comparable experiments, C 17 and C 18 in Table V.) But with regard to creatinine the kidneys were working excellently and taking 15 mg. of this substance from every 100 c.c. of blood; it can thus not be considered that there was any general depression of renal function.

The interference with the renal extraction ratios for urea leads, as would be expected, to some elevation of the blood urea. In five rabbits the blood urea was measured immediately before and at 4 hours after an intravenous injection of 2000 mg. of creatinine per kg.; the dose was the same as that used in series G. The results are given in Table XVI; it will be seen that there is an average increase of nearly 10 mg. urea per 100 c.c. at 4 hours after administering the creatinine.

TABLE XVI. Effect of large doses of creatinine on blood urea.

Rabbit	Blood urea mg. per 100 c.c.	
	Before creatinine injection	4 hours after creatinine injection
H 1	29.4	37.6
H 2	38.9	51.2
H 3	39.4	50.2
H 4	48.2	55.0
H 5	48.4	58.2
Mean	40.8	50.5

## PLASMA RENAL EXTRACTION RATIOS.

## CREATININE.

The discussion so far has taken no account of the relative amounts of creatinine in the plasma and in the corpuscles. For the purpose of simplicity all renal extraction ratios have been calculated as those for whole blood. It is now necessary to consider a complicating factor of definite and fundamental importance.

The creatinine in the blood is not carried in the plasma alone. Part of it is in the corpuscles; presumably it has entered them by a process of diffusion. For practical purposes the creatinine in the corpuscles is outside the immediate scope of the activity of the kidney. This follows from the following considerations. The initial diffusion from the plasma to corpuscles is not very rapid; at 1 min. after the injection the corpuscles contain only 20 p.c. of the total creatinine in the blood, though later they will contain 40 p.c. During the first minute the conditions favouring diffusion are very good, the plasma having a high creatinine content and the corpuscles a very low one. This may be contrasted with the condition of the blood which is exposed to the action of the kidney a few minutes later. At that time an equilibrium has been reached with the percentage of creatinine in the plasma about equal to that in the corpuscles. If the kidney suddenly removes 20 p.c. of the creatinine in the plasma passing through the renal capillaries, the equilibrium will be disturbed and a

return diffusion of creatinine from corpuscles to plasma will take place. It is, however, most unlikely that such a return diffusion can reach any significant proportion during the 3 or 4 sec. that the blood is passing through the renal capillaries, since the known rate of diffusion during 60 sec. is, as shown above, not really very great even under very favourable conditions. Probably, therefore, the creatinine in the corpuscles passes through the renal capillaries practically unaltered in amount.

The significant figure in considering the renal elimination of creatinine is, therefore, the amount of creatinine in the plasma going to the kidney, since only this amount of creatinine is exposed to the activity of the kidney. The figures given in an earlier section may be summarized as follows. At the time of the injection the plasma contains practically all the creatinine in the whole blood; at the end of 1 min. it contains 80 p.c. of it. An average figure of at least 90 p.c. in the plasma may be taken as representing approximately the condition during the first minute. On the other hand, after the first minute the plasma contains 60 p.c. of the creatinine in the whole blood.

The bearing of these points on the renal extraction ratios is that the kidney is not taking up creatinine from the whole amount in the blood but only from the part of it in the plasma. The conception of a "plasma renal extraction ratio" must be added to that of the ordinary "whole blood renal extraction ratio." The plasma renal extraction ratio is the true measure of renal elimination, since the ordinary ratio includes creatinine which is not exposed to the activity of the kidney. The calculation of the plasma renal extraction ratio for any individual experiment depends of course on the proportion of creatinine in the plasma having been ascertained exactly, but the average ratios for the whole series of experiments can be calculated with sufficient accuracy by multiplying the ordinary ratios during the first minute by 100/90 and those after the first minute by 100/60.

Without going into the figures of individual experiments the following averages may be given:

	Whole blood renal extraction ratio p.c.	Plasma renal extraction ratio p.c.
During first minute after injection	28	31
More than 24 min. after injection:		
(a) At blood creatinine of about 20 mg. per 100 c.c.	23	38
(b) At blood creatinine of about 100 mg. per 100 c.c.	14	23
(c) At blood creatinine of about 200 mg. per 100 c.c.	8	13

It should be explained at this point that the distribution of creatinine in the blood between plasma and corpuscles has not been studied in every experiment. For this reason the matter has not been raised earlier, since it would have involved expressing certain results as plasma renal extraction ratios and others as whole blood renal extraction ratios. The general level of the plasma renal extraction ratios can, however, be established quite satisfactorily by the method here adopted. In the individual experiments where the actual plasma-corpuscle proportion of creatinine was measured, the plasma renal extraction ratios are in agreement with the figures calculated for the whole series of experiments.

#### UREA.

Unfortunately no actual measurements were made of the distribution of urea between corpuscles and plasma in the present work; the amount of apparatus required made such additional investigations impracticable. Certain conclusions, however, may legitimately be drawn from the facts which are known.

It has been established by many workers that the urea content of the corpuscles is the same as that of the plasma, whatever the level of the blood urea. Thus at several minutes after the injection of urea the same condition may be presumed to obtain; the plasma therefore contains 60 p.c. of the whole amount of urea in the blood. The only question which requires more detailed consideration is the rate at which this equilibrium is reached. Some indirect information on the point can be obtained from a comparison of the rates of disappearance of urea and creatinine from the heart blood. The following figures represent the approximate amounts in the heart blood at two intervals after the intravenous injection of 1000 mg. of the substance per kg.

	Urea mg. per 100 c.c. blood	Creatinine mg. per 100 c.c. blood
5 min. after injection	170	280
50     "     "	140	140

It will be seen that both substances reach the same level in the blood after 50 min., but that the initial rate of disappearance of urea is much greater. The disappearance is due chiefly to the passage of the substance into various tissues of the body. It would therefore appear that urea can pass more readily from the blood into the tissues than creatinine. Probably the same rule applies to the passage from plasma to corpuscles. It seems therefore permissible to assume that the mean proportion is 80 p.c. for urea as against 90 p.c. for creatinine. As indicated above,

a proportion of 60 p.c. of the urea in the plasma can be accepted as quite accurate for experiments several minutes after the injection.

The following plasma renal extraction ratios are calculated by multiplying the average ordinary renal extraction ratios during the first minute by 100/80 and those after the first minute by 100/60.

	Whole blood renal extraction ratio p.c.	Plasma renal extraction ratio p.c.
During first minute after injection	32	40
More than 50 min. after injection:		
(a) At blood urea of about 30 mg. per 100 c.c.	9	15
(b) At blood urea of about 100 mg. per 100 c.c.	7	12
(c) At blood urea of about 200 mg. per 100 c.c.	5	8

[(a) includes the figures of Dunn, Kay and Sheehan on normal rabbits.]

#### INTERPRETATION OF THE RENAL EXTRACTION RATIOS.

To avoid misunderstanding it is necessary to define certain words used in this section. *Reabsorption* means the passage of a substance from tubular lumen to tubular epithelium; *direct absorption* means the passage from the blood in the intertubular capillaries to the tubular epithelium. *Secondary return* means the passage from tubular epithelium to the blood in the intertubular capillaries; *secretion* means the passage from tubular epithelium to tubular lumen. A substance may enter the tubular epithelium by either of the first two methods, and leave it by either of the second two methods; e.g. there is no fundamental reason why direct absorption should be followed by secretion rather than by secondary return. Furthermore, a substance entering the tubular epithelium by either method may leave it at once, or only after an interval, or may be stored or metabolized there. Of course, none of these processes comes into play in the case of a substance which is eliminated from the blood by filtration alone and which passes down the tubule without any reabsorption.

The present work deals merely with the elimination of urea and creatinine from the blood by the kidney. This is not to be confused with the excretion of these substances in the urine. The two processes must finally balance; there is certainly not a permanent accumulation of the substances in the kidney. But there is no evidence that each unit of urea or creatinine removed by the kidney from the blood is at once excreted into the urine. The significance of this distinction has been the subject of discussion elsewhere [Ekehorn, 1932; Sheehan, 1932 b, 1933].

It may be mentioned at this point that, though the kidney may possibly produce or utilize creatinine or urea, such processes can hardly play any significant part in the present experiments, in view of the relatively large amounts of the two substances which have been administered.

The essential results of the whole investigation are summarized below. The figures given are arithmetical averages, but they are given here only because they indicate the general level of the ratios. Any single experimental result represents only a brief incident in the renal activity of one animal; yet, taken all together, the results obtained from many animals under different experimental conditions as to time, plasma concentration, etc. may reasonably be considered to indicate the usual renal response of any animal to the particular substance injected. The results are calculated to give the proportion of the substance which is removed from the plasma during its passage through the kidney.

	Plasma renal extraction ratio p.c.	
	Urea	Creatinine
During first minute after injection	40 (maximum 50)	31 (maximum 43)
At 2-20 min. after injection	2	19
More than 50 min. after injection:		
(a) At blood content of about 20 mg. per 100 c.c.	15	38
(b) At blood content of about 100 mg. per 100 c.c.	12	23
(c) At blood content of about 200 mg. per 100 c.c.	8	13

The next step is to consider how these figures are to be interpreted in terms of renal function.

#### RATIOS LATER THAN 50 MIN. AFTER THE INJECTION.

The chief problem that arises is the explanation of the inverse relationship between the amount of creatinine or urea in the blood and the renal extraction ratios for these substances, in the experiments after the early "dip" in the graphs has passed off. Associated with this is the problem of the renal extraction ratios found in the combined experiments.

The former question will be considered first in the case of each substance.

##### *Creatinine.*

The "filtration" theory affords two possible explanations, (a) decreased filtration, or (b) increased reabsorption and secondary return at high blood creatinines.

*Decreased filtration.*

It is exceedingly improbable that the large amount of creatinine in the blood could provide any physical impediment to filtration of the type exerted by proteins. The only reasonable theory is that the high blood creatinine interferes with filtration by producing vasomotor effects on the glomerular arterioles or capillaries. Although this need not necessarily be associated with any alteration of renal blood flow, it is of interest to note the low renal blood flows after the administration of very large amounts of creatinine in C 17 and C 18 and G 1-G 4. This vasomotor disturbance is not directly related to the height of the blood creatinine as is seen from experiments C 1-C 15. Nevertheless, the occurrence of vasomotor effects in the former cases makes the occurrence of other types of vasomotor effects a distinct possibility.

This theory provides, however, no plausible reason for the almost complete arrest of urea elimination in series G.

*Increased reabsorption and secondary return.*

At first sight it appears rather paradoxical that reabsorption and secondary return should increase when the blood creatinine is very high, and therefore when the animal is in most need of excreting the substance. Two points must, however, be borne in mind:

(1) The increased reabsorption and secondary return are only relative. The absolute difference between the creatinine in heart blood and renal vein blood does become greater as the blood creatinine rises; the problem at issue is why this difference becomes a steadily diminishing proportion of the blood creatinine.

(2) Reabsorption and secondary return are not necessarily active functions of the tubular epithelium; they may represent only a passive diffusion from the tubular lumen back to the blood. Now there is a maximum concentrating power of the kidney as a whole for different substances; *e.g.* from estimations on bladder urine it is found that the rabbit concentrates urea only up to about 5 p.c., the cat up to about 10 p.c. There is presumably therefore a maximum concentration in the renal tubules which is specific for each substance. Information as to the exact level of this maximum concentration in the different parts of the tubule is unobtainable. But when the blood creatinine is very high a relatively smaller removal of water from the filtrate may raise the creatinine in the tubular urine to near its maximum concentration. In this case the tubular epithelium will be under steadily increasing strain. It may respond either by reabsorption and secondary return of creatinine



(either as an active process or merely by allowing diffusion) or by removing less water from the tubular urine. The former would provide a reasonable explanation of increasing reabsorption and secondary return with increase of the blood creatinine and thus for a diminishing renal extraction ratio.

Furthermore, if the tubular epithelium is under heavy strain in dealing with creatinine, it seems possible that it might fail also to retain urea efficiently in the tubule, even though the urea was not in high concentration.

According to the "secretion" theory it would be expected that the tubular epithelium would be stimulated to absorb directly and secrete a greater absolute amount of creatinine the higher the blood creatinine. In opposition to this, the greater the absolute amount of creatinine secreted the greater would be the work done by the tubular epithelium, and therefore presumably the less efficiently would this epithelium deal with additional work. In this connection, the available supply of oxygen might be a factor of importance, but this is still undecided. Gremels [1930] found that the oxygen usage of the kidney is roughly proportional to the amount of excretion of nitrogenous substances; on the other hand Rhoads, van Slyke, Hiller and Alving [1931] found that the oxygen consumption of the kidney is not markedly affected by increasing the urea excretion.

If the tubular epithelium were under heavy load in directly absorbing and secreting creatinine, it is of course quite possible that it would be unable to deal with urea efficiently, and thus that the renal extraction ratio for urea would be lowered when the blood creatinine was very high, as in series G.

#### *Urea.*

The same explanations apply to the urea results as to the creatinine results.

The sudden reduction of renal blood flow during the injection in the long sample experiments is evidence that urea can produce vasomotor disturbance. The possibility thus arises that it may affect the vasomotor control of filtration in all the experiments. This, however, does not explain the fact that the renal extraction ratios for creatinine in series F were not affected by the high blood urea. If the amount of filtration were the controlling factor, a reduction in the percentage of plasma filtered should affect the renal extraction ratio of the two substances equally.

The occurrence of increased reabsorption and secondary return when the blood urea is high would provide a more satisfactory explanation.

The fact that the renal extraction ratio for urea is lower than that for creatinine means, according to a pure "filtration" theory, that much of the filtered urea is normally reabsorbed into tubular epithelium and secondarily returned to the blood. This process may be presumed to occur fairly easily, and apparently is capable of increase without necessarily involving the reabsorption and secondary return of creatinine.

On the other hand, a "secretion" theory offers a plausible explanation. As in the case of creatinine, the gradual diminution of the renal extraction ratios with rising blood urea is to be expected, as the work done increases with increase of the absolute elimination of urea from each 100 c.c. of blood. An easy solution can also be given for the problem of series F and G.

By reference to the average "differences" in these two series tabulated earlier, it will be seen that in series F the absolute amount of urea eliminated from the blood by the kidney was about 4 mg. per 100 c.c. more than normal. This cannot be considered a serious overload for the kidney which has normally to eliminate 2.5 mg. of urea from each 100 c.c. of blood and can remove as much as 15 mg. per 100 c.c. The kidney was also able to eliminate at the same time over 2 mg. per 100 c.c. more creatinine than normal without any evidence of failure.

On the other hand, in series G the kidney was eliminating about 15 mg. creatinine more than usual from each 100 c.c. blood, which is about a hundred times the normal amount. This may be considered to be probably a big load for the tubular epithelium, the highest "difference" observed in any experiment being 18 mg. per 100 c.c. With this work to be done in dealing with creatinine, it is hardly surprising that the kidney practically ceased work with regard to urea.

The inverse relationship between the height of the blood urea or creatinine and the renal extraction ratio for these substances can thus be explained sufficiently by either a "filtration" theory or a "secretion" theory. From the point of view of the former theory, it appears more probable that reabsorption and secondary return are increased than that filtration is diminished when the blood urea or creatinine are raised.

The results of the experiments where both substances were injected together can also be explained by either theory, but are more readily explainable on the view that the elimination of the substances is an active process.

## RATIOS DURING THE FIRST MINUTE AFTER THE INJECTION.

The ratios for creatinine and urea found in the long sample experiments can be explained fairly easily by a "filtration" theory. It is necessary to assume that about 30-40 p.c. of the plasma is filtered in the glomeruli and that, even if reabsorption of urea or creatinine into tubular epithelium does occur during the first minute, there is no secondary return of the substances to the blood during this time. The plasma renal extraction ratios are thus about maximal, and give a direct measure of the percentage of plasma filtered. The fact that the ratios for urea are higher than those for creatinine during the first minute is explicable on the view that the vasomotor disturbance produced by the injection of the urea may increase the amount of filtration to as much as 50 p.c. of the plasma entering the renal blood vessels.

The ratios can, however, be equally well explained as due to the direct absorption of creatinine and urea from the blood by the tubular epithelium. This epithelium contains a relatively small amount of the substance at the start of the experiment. Suddenly the blood in the renal capillaries comes to contain a large amount of the substance. It seems not unlikely that the tubular epithelium should take up the creatinine or urea rapidly until a state of equilibrium is reached between the epithelium and the blood. This direct absorption may be either an active process on the part of the cells or merely diffusion of the substance, or both.

The view that the kidney absorbs creatinine and urea directly from the blood during the first minute is supported by two facts. First, the tubule cells certainly present to the blood stream a surface capable of direct absorption, as has been shown previously in the case of dyes. Second, the urea and creatinine are removed from the blood by many other tissues during the first minute after injection, as is obvious from Figs. 8 and 9 (which are given later); this can only be by direct absorption of the urea and creatinine by these tissues. The kidneys take up a bigger proportionate amount of these substances than the average for the rest of the body. There do not seem to be satisfactory grounds for denying to the kidneys a property which is possessed by most other tissues in the body excluding fat and bones [Marshall and Davis, 1914].

Comparison of these two figures shows also that urea is removed from the general blood stream more rapidly during the first few minutes than is creatinine. The higher renal extraction ratio for urea than for creatinine indicates that the kidney is, like the other tissues of the body,

able to remove urea more easily than creatinine from the blood during the first minute after injection. A similarity in the method of removal of the substances by the kidney and by other tissues is certainly suggested.

The further question arises as to why the ratios found in the long sample experiments tend to be higher when the collection of renal vein blood is short than when it is long. On the basis of a "filtration" theory, if the amount of filtration remains fairly constant, it is necessary to assume a rather marked loss of creatinine (or urea) from the kidney in the second half minute during which renal vein blood is being collected. Such loss might be by excretion into the bladder, or by reabsorption and secondary return to the blood stream. As is discussed later, it seems unlikely that any significant loss by excretion can occur; the possibility that reabsorption and secondary return may be in progress remains.

On the other hand, the facts can be easily explained on the view that the early rapid accumulation of creatinine occurs by direct absorption. During the initial peak of high creatinine in the blood the kidney takes up a great deal of creatinine from the blood. There is then a sudden fall in the creatinine content of the blood. The conditions which led to the initial rapid direct absorption are thus reversed in the latter half minute of the blood collection, and it is therefore to be expected that direct absorption will be much reduced or perhaps even replaced by secondary return.

The same explanations which have been given for the creatinine ratios apply of course equally to urea.

#### RATIOS AT 2 TO 20 MIN. AFTER THE INJECTION.

The dip in the extraction curves of both urea and creatinine during the first 20 min. follows on the rapid taking up of these substances by the kidney, and occurs at a time when their concentration in the blood has fallen much below its peak level. According to either theory a negative extraction ratio is undoubted evidence of secondary return. In the urea experiments there is therefore proof that urea sometimes passes from the tubular epithelium into the blood. This cannot be interpreted as indicating by which route this urea had entered the tubular epithelium. Rhoads, van Slyke, Hiller and Alving [1931] remark that "the occurrence of renal blood urea contents markedly higher at times than arterial urea contents appears to be proof of the occurrence of reabsorption of urea from the kidneys." Their conclusion is quite justified, so long as their term "reabsorption" is understood to refer only to what

has been called here "secondary return" of urea from the kidneys to the blood. It must obviously not be extended to include "reabsorption" of urea from tubular urine into the kidney parenchyma.

Explained by a "filtration theory," a negative extraction ratio would indicate that more urea is being reabsorbed and secondarily returned to the blood than is being removed from the blood by filtration. In this connection two points must be recognized. First, the time required for reabsorbed urea to pass across the epithelial cells and be returned to the blood is quite unknown; the process may be almost immediate or take a long time. Second, the tubular urine contains much urea which could act as a supply for reabsorption and secondary return apart from urea in the fluid which is actually being filtered during the time of the experiment, or urea which is present in tubular epithelium.

According to a "secretion" theory the secondary return of urea which has been directly absorbed earlier would indicate that the high-level equilibrium between tubular epithelium and blood, reached within a few seconds after the injection, has become disturbed by the fall in the blood urea. This explanation of a period of readjustment is the same as that suggested earlier to account for the lower ratios in long sample experiments that continue about a minute. The question immediately arises—why should a secreting epithelium regain its equilibrium by returning urea to the blood instead of by secreting the urea into the tubular lumen? This objection is not as serious as it appears on first glance. In these experiments the kidney receives a sudden large dose of urea; excretion certainly begins fairly quickly, and probably not many minutes will pass before the tubular urine contains about its maximum percentage of urea. As a result, the secretion of urea is presumably partially inhibited, and the tubular epithelium, overloaded with urea, finds relief by secondary return as well as by what secretion it still remains capable of.

It must of course be understood that the occurrence of a negative renal extraction ratio for urea does not imply that the kidney is not excreting urea at the time. The processes of secretion and secondary return are not mutually exclusive; they may quite well occur simultaneously.

The renal extraction ratios which are low but still positive come into the same theoretical discussion; they include most of the urea experiments and all of the creatinine experiments at the time of the dip in the curves. According to a "filtration" theory, the renal extraction ratio indicates the combined effect of the filtration and of the secondary return of reabsorbed substance; according to a "secretion" theory it indicates

the balance between direct absorption and any secondary return which may occur. The two postulated activities partially counterbalance each other at the "dip" period, and the result is either a low renal extraction ratio or sometimes even a negative one according to whether the balance is a little net filtration or direct absorption or a net secondary return.

Why then is the dip in the urea curve so much longer than that in the creatinine curve? The creatinine in the blood falls more rapidly than urea between 3 and 50 min.; according to a "secretion" theory this should lead to greater disturbance of equilibrium between the creatinine content of the tubular epithelium and that of the blood than in the case of urea. The chief difficulty in answering the question lies in the complete absence of knowledge as to what are the maximum possible percentages of urea and of creatinine in the urine in the different parts of the tubules. It is thus not possible to discuss the relative difficulty in the secretion of the two substances by the tubular epithelium.

#### GENERAL.

If the results are explained on the basis of a pure "filtration" theory, the plasma renal extraction ratios represent the minimum amount of filtration that occurs. Should any secondary return occur, the amount of filtration must of course be higher. The plasma renal extraction ratios for small quantities of creatinine 1 or 2 hours after the injection are about 40 p.c.; those for urea are about 15 p.c. These figures agree quite well with the relative levels of the plasma clearances found by other investigators [Rehberg, 1926; Cope, 1931; Jolliffe and Smith, 1931].

On the view that creatinine is not directly absorbed from the blood the results would indicate that about 40 p.c. of the plasma is filtered off in the glomeruli and that two-thirds of the filtered urea is reabsorbed and secondarily returned to the blood. The lower ratios in many experiments with creatinine require the assumption either that this substance can sometimes be reabsorbed or that the filtration of plasma in these cases was below 40 p.c. The plasma renal extraction ratios for urea during the first minute after its injection are sometimes as high as 50 p.c.; this requires either that some urea is directly absorbed or that the filtration of plasma was as much as 50 p.c.

On the other hand, if direct absorption of urea or creatinine does occur, all the results can be explained by this process alone. If direct absorption be responsible for only part of the elimination of these substances from the blood, some lower amount of filtration than 40 p.c.

may be postulated, and the higher ratios are then a measure of the amount of direct absorption.

From the experimental results it is not possible to adduce any single fact in absolute proof or disproof of either the "filtration" or the "secretion" theory of renal function. A plasma renal extraction ratio of 5 p.c., such as occurs in some of the urea experiments, is certainly low enough to be explained on a simple "filtration" theory. On the other hand, a ratio of 100 p.c., such as is found with certain dyes, is obviously too high to be explained in this way. The ratio must at some level between these two extremes become too high to be accounted for by "filtration" alone; at what point remains an unsolved problem. Although the filtration of half the plasma in the glomeruli as suggested by some of the urea long sample experiments is certainly rather high, it is not in any way impossible on theoretical grounds. Ekehorn [1931] considers it probable that 25-30 p.c. of the blood passing through the glomerular tufts is filtered off; these figures correspond to about half the plasma.

#### APPENDIX.

##### CONSIDERATION OF EXPERIMENTAL TECHNIQUE.

###### *Long sample experiment.*

The methods of obviating significant errors require brief consideration. The criteria to be given of a satisfactory experiment are necessarily set at arbitrary levels, but they are sufficiently stringent to reduce the possible errors to unimportant proportions. For purposes of clarity, creatinine alone is discussed below. Except where specific differences are pointed out it is to be understood that the discussion applies equally to urea.

###### *Endogenous creatinine and urea.*

The renal vein blood contains, in addition to the injected creatinine, a certain amount of endogenous creatinine. This source of error is negligible if the amount of creatinine injected be large; *e.g.* about 100 times the amount normally in the animal's whole blood volume. The actual amount of creatinine injected was always over 100 mg. per kg. of body weight.

An allowance is, however, required for the endogenous creatinine contained in the renal parenchyma and in the urine in the kidney. This amount is relatively not very large because of the diuretic action of the urethane, and also because most of the urine in the renal pelvis escapes when the kidney is taken out of the body and while the perirenal fat

is being removed from it. The necessary correction for the endogenous renal creatinine was found from a series of control experiments on anaesthetized animals which received no injection of creatinine. In order that the correction might be relatively small, the amount of creatinine injected in the long sample experiment was so adjusted that the amount taken up by the kidney was at least three times as much as the average amount in the kidney before experiment. For this purpose the amount injected had to be over 300 times as much as the amount in the kidney before experiment.

It will be seen from Table XVII that the allowance for endogenous creatinine in a kidney at the beginning of a long sample experiment may be taken as 0.4 mg. In order that this should not be of too much importance the amount of injected creatinine taken up by the kidney during the experiment should be over 1.2 mg.; the total amount of creatinine injected should therefore be at least 120 mg.

TABLE XVII. Amounts of creatinine and urea in the kidneys of control rabbits killed half an hour after anaesthetization with intravenous urethane.

Rabbit	Creatinine (mg.) in	
	Left kidney	Right kidney
I 1	0.19	0.17
I 2	Not estimated	0.18
I 3	"	0.19
I 4	0.22	0.21
I 5	0.26	0.21
I 6	0.24	0.28
I 7	0.56	0.53
I 8	0.65	0.56

Mean creatinine per kidney 0.32 mg.

	Urea (mg.) in	
	Left kidney	Right kidney
J 1	5.9	Not estimated
J 2	7.8	6.9
J 3	8.7	9.8
J 4	9.4	Not estimated
J 5	11.1	10.4

Mean urea per kidney 8.8 mg.

Similarly in the case of urea, as shown in Table XVII, the mean amount of endogenous urea in the kidney is 8.8 mg.; the amount of injected urea taken up by the kidney during the experiment should therefore be over 24 mg.; the total amount to be injected is thus at least 2400 mg.



*Injected creatinine and urea.*

(1) To ensure that the wave of high creatinine content in the blood had passed through the kidney to the renal vein, the following conditions were observed.

(a) The renal vein blood should be collected for more than 25 sec. after the beginning of the injection into the ear vein, and for more than 10 sec. after the end of that injection.

(b) At least 5 c.c. of blood should be collected from the renal vein subsequent to 10 sec. after the beginning of the injection.

In the case of urea, in view of the unevenness of the circulation rates, it is more important than ever that the total renal blood flow should be sufficient for the peak of the urea content of the blood to have passed through the kidney to the renal vein. The peak is longer and lower owing to the length of the injection. Therefore, in addition to the ordinary criteria of long sample experiments, a further standard has been set up; the blood collected after the end of the injection of urea must be at least 2.5 c.c.

(c) The renal circulation rate should be over 0.75 c.c. per g. per min.

(d) The amount of creatinine reaching the operated kidney *via* the renal artery during the experiment should be at least 2.5 p.c. of the amount injected.

(e) There should be no evidence that the blood flow to the operated kidney had been affected by any local reflex from the operation; for this, the amount of creatinine taken up by the left kidney, should preferably be within  $\pm 10$  p.c. of that taken up by the right kidney. The collection of the renal vein blood must of course be perfectly free and unobstructed.

(2) For the present purpose the urine in the tubules was considered to be in the kidney proper, whereas the blood in the renal capillaries was not. There was some escape of blood and urine from the hilum of the kidney after removal and during the process of cleaning.

(a) Hæmoglobin estimations of aqueous kidney extracts showed that the blood remaining in the kidney was very rarely as much as 0.2 c.c. If this blood had the same creatinine content as the renal vein blood, it would raise the amount in the kidney by 1 or 2 p.c. of the observed amount; the effect of this on the renal extraction ratio is negligible.

(b) It is unlikely that much of the injected creatinine which had been taken up by the kidney was lost from the hilum; the experiments were not very long, and any injected creatinine in tubular urine was probably not in the collecting tubules in any quantity at the end of the collection

of renal blood. If loss did occur it would lead to under-estimation of the amount of creatinine taken up by the kidney, and thus to under-estimation of the renal extraction ratio.

(3) The long sample experiments can only give average values for the renal extraction ratio during the whole minute that the experiment lasts. It is quite possible that the ratio is high at 10–20 sec. after the beginning of the injection and then becomes smaller. It is also possible that, as the creatinine is rapidly disappearing from the blood, there may be a secondary return of creatinine from the kidney to the blood towards the end of the experiment. Any such secondary return is, however, not to be looked on as an error leading to under-estimation of the net amount of creatinine taken up by the kidney, but as an integral part of this net amount.

*Short sample experiment.*

The short sample experiment consists essentially of determining small differences of concentration of creatinine between the heart blood and renal vein blood. The differences are so small that very slight errors are of far more serious significance than in the long sample experiments. A few points thus require detailed consideration.

*Renal vein blood sample.*

(1) Creatinine is a normal constituent of blood and urine. The blood normally contains a small amount of "chromogenic substance" [see Hunter, 1928] which is not creatinine. The actual amount is uncertain, but it is probably large enough in proportion to the normal creatinine content of the blood to introduce gross errors in the determination of the renal extraction ratio in an animal whose blood creatinine is at the normal level.

To render the error due to "chromogenic substances" of no significance, and to permit of accurate estimation of the blood creatinine, it is necessary to raise the blood creatinine artificially, preferably to about twenty times the normal.

This difficulty does not arise in the estimation of the renal extraction ratio for urea at its normal level.

(2) As the differences between the creatinine contents of the heart blood and renal vein blood were very small, the slightest reflex interference with kidney function at the time of the operation might have completely upset the results of any experiment. This matter is very important [see Addis and Shevky, 1917; Nash, 1922]. Much of the older work, and even some more recent, on renal vein blood chemistry

has been vitiated by such reflexes. The only direct information about the question of whether local reflex disturbance of renal function occurs in the operated kidney is obtained from the long sample experiments. As has been shown the agreement between the amounts of creatinine taken up by the two kidneys in those experiments suggests that no such reflex develops. This conclusion may, however, only be transferred to the short sample experiments with the greatest caution. The condition of the kidneys in the two types of experiment is in no way analogous. At the beginning of a long sample experiment the kidney contains only very little creatinine and is suddenly confronted with a large amount in the blood coming to it. Under such a strong stimulus a very gross reflex would presumably be required to inhibit the kidney from dealing with the creatinine. On the other hand, at the beginning of a short sample experiment some minutes after the injection of the creatinine the kidney contains a great deal of creatinine; the amount in the blood is steadily falling. In these short sample experiments the balance between the amount of creatinine in the kidney and that in the blood may be fairly delicately adjusted; if so, a slight reflex disturbance from the operation might upset the balance. The evidence from the long sample experiments cannot therefore be accepted as a definite indication that disturbances of renal function do not occur as a reflex from the operation.

A further point, however, requires consideration. If, owing to the operation, local reflexes develop, they will probably be multiple in type; *i.e.* disturbance of renal function will probably be associated with disturbance of renal circulation, though both may be independent reflexes. Such vasomotor alterations may be expected to be constrictor, from the known facts about the influence of grosser operative interference on the deposition of dyes in the kidney. Thus a slowing down of the renal blood flow during the operation is possibly an indication of reflex disturbance of renal function. A slight slowing down was, however, only seen three times in the present series of experiments and the renal extraction ratios were not abnormal in these cases. Further, the evidence of the long sample experiment shows that the present method of collecting blood from the renal vein does not produce any significant difference between the blood flow to the two kidneys.

It therefore appears highly probable that no special local reflexes occurred in the operated kidney, either vascular or functional. Nevertheless, in order to reduce the possibility of any such reflex developing, the collection of renal vein blood was made as short as possible. Certain criteria of a "satisfactory" experiment were adopted, as in the long

sample experiment. The exact figures are somewhat arbitrary but in practice give a reasonable dividing line between short sample experiments which were really short and those which were unsatisfactorily prolonged; most experiments either fulfilled the required conditions easily, or failed even to approach them.

(a) The 3 c.c. of blood required for creatinine estimations must be collected within 30 sec.; the 5 c.c. of blood for urea estimations within 40 sec.

(b) The renal circulation rate must be over 0.75 c.c. per g. per min.

(3) Certain of the experimental results raise the question as to whether too forcible aspiration of renal vein blood might lower the pressure in the renal venules so much as to produce pyelo-venous back flow [Hinman and Lee-Brown, 1924]. This does not appear possible. If the suction is too great the wall of the renal vein is drawn down, as a valve on the bevel of the needle point; it is thus impossible to produce a negative pressure in the renal venules. Further, accidental pyelo-venous back flow would lead to widely varying and inexplicable results, whereas the variations in the actual experiments were related to methodical and ascertainable factors. This problem can thus not be considered as of any significance.

#### *Heart blood sample.*

The application of a correction to the heart blood sample to allow for the disappearance of creatinine from the heart blood during the experiment is a matter more of theoretical than of practical importance. In the case of dyes where the short sample experiments were performed within a few seconds after the injection this correction was of great significance. In the present experiments it is only in the experiments performed within a few minutes after the injection that it is large enough to require consideration.

It is required to know the creatinine content of that blood which was in the heart about 3 sec. before the mid-point of the collection of the renal vein sample, 3 sec. being approximately the time of circulation of the blood from the heart to the renal vein. The heart blood sample is actually obtained at some time later, usually from 8 to 24 sec. after the mid-point of the collection of the renal vein sample. The amount of the correction depends on two numerical factors: (a) the interval elapsing between the collection of the two samples of blood; this was measured in the experiment, and (b) the rate of disappearance of the creatinine from the heart blood at the time of the experiment. This rate can be

ascertained by comparing the amounts of creatinine in the heart bloods of a large number of rabbits at known intervals after the intravenous injection of a standard amount of creatinine, and estimating from this the average rate at which the creatinine disappeared from the blood in the whole series of rabbits. This average rate corresponds to what was found by making in a few individual rabbits two measurements of the creatinine content of the blood with an interval of several minutes. The method described above is necessary to determine the rate of disap-

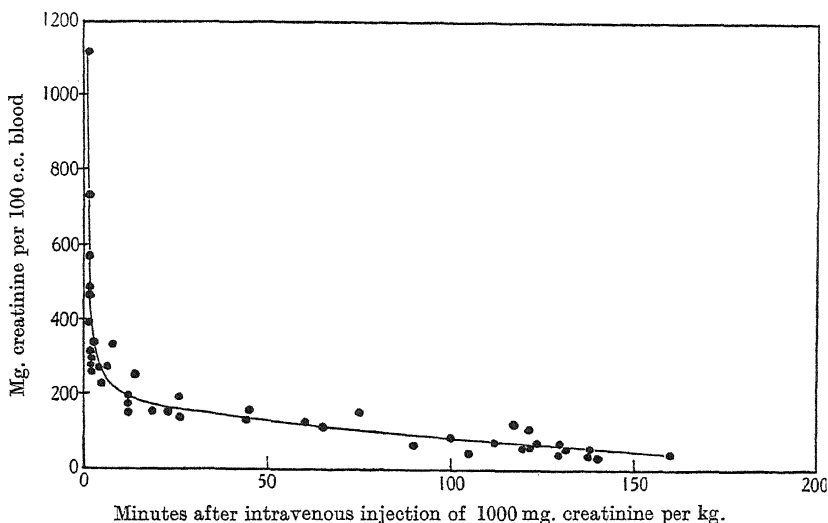


Fig. 8. Rate of disappearance of creatinine from blood.

pearance, as a large series of determinations at very short intervals of the creatinine content of the blood of a single rabbit is unsatisfactory. It was shown [Sheehan, 1931] that this procedure produces a quite abnormal rate of disappearance of substances from the blood.

The rate of disappearance of injected creatinine from the general blood stream is illustrated in Fig. 8. The points indicate the amount of creatinine per 100 c.c. heart blood of individual rabbits operated on at different intervals after the intravenous injection of creatinine. Most of the animals had received only 200–500 mg. of creatinine per kg., but the figures have been calculated to a standard dosage of 1000 mg. per kg. so that Figs. 8 and 9 may be comparable. There is no significant difference between the rates of disappearance of the creatinine from the blood after the small or the large dosage. In only five animals was the creatinine

in the heart blood less than 25 mg. per 100 c.c.; the pre-existent creatinine in the blood is thus relatively so small as to require no allowance in calculations.

An animal which has 100 c.c. of blood weighs about 1.5–2 kg.; on a dosage of 1000 mg. per kg. it would therefore receive 1500–2000 mg. creatinine into its 100 c.c. of blood. The initial level of the creatinine in the blood immediately after the injection would theoretically be about 2000 mg. per 100 c.c. On extending the area of the graph to include this it will be seen that the creatinine disappears from the blood in two chief phases; rapidly during the first 2 or 3 min., and slowly after this time.

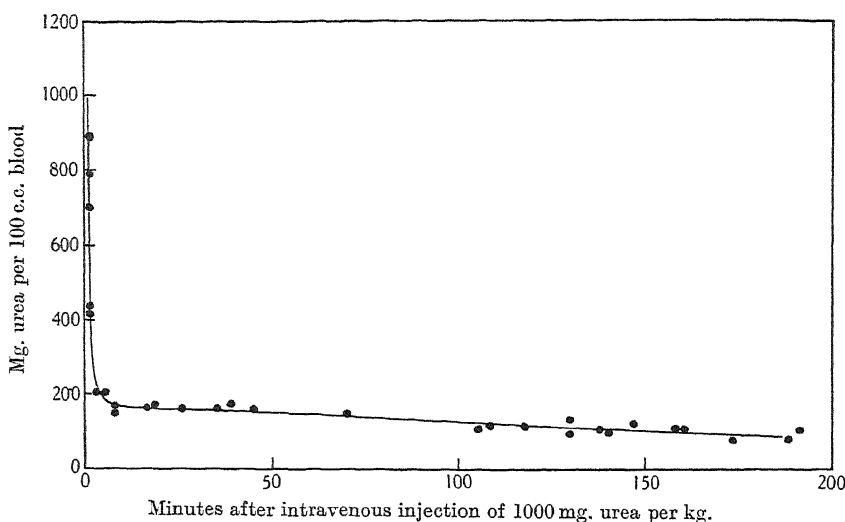


Fig. 9. Rate of disappearance of urea from blood.

In the case of urea various doses were injected. The adjustment of the blood ureas to what they would have been after the injection of a standard dose is interfered with by the need for a correction for urea pre-existent in the blood. This was not measured in the present experiments. In an earlier series of experiments it lay between 16 and 47 mg. per 100 c.c.; this range is too wide to allow of an average figure being adopted, in view of the relatively large size of the correction in cases where only small amounts of urea were injected.

In order to avoid the mathematical multiplication of figures, with the associated multiplication of correction errors, the points given in Fig. 9 are taken only from experiments where 1000 mg. of urea were

injected per kg., except for the figures at 1 min. Here the amount injected was from 1250 to 2500 mg. per kg.; the figures are so high that no correction is required, and the calculation to standard dosage is a division.

The blood urea at the end of the injection is probably about 1500–2000 mg. per 100 c.c. It will be seen that, like creatinine, there is a very rapid fall in the graph during the first 2 or 3 min. (probably due to the passage of urea into various tissues of the body) and subsequently a very slow fall (probably related to excretion).

It is difficult to estimate with any degree of accuracy the rate at which the urea and creatinine disappear from the blood during the first 10 min.; the following figures are only rough approximations.

Time after injection of creatinine or urea min.	Rate of disappearance. Percentage of actual amount in blood disappearing from blood per min.	
	Creatinine	Urea
3	15	5
7	4	1
10	3	0.5

In the majority of the experiments during the first 10 min. after injection, the intervals between the mid-points of the collections of renal vein and heart blood samples were from 6 to 11 sec. Most of the corrections of the heart blood figures are thus calculated from only 1/10 to 1/6 of the above rates of disappearance. The effect of the corrections on the renal extraction ratios is not to raise any of them by more than 1 p.c. except in one single case, rabbit C 1, where the correction raises the renal extraction ratio by 6 p.c. The corrections other than C 1 have been ignored as negligible.

#### *The effect of urea on peritoneal absorption.*

In four of the urea experiments an attempt was made to stimulate the renal function by giving an intraperitoneal injection of 50 c.c. of physiologically normal saline about an hour before the operation, but after the intravenous injection of the urea. A rather unexpected result was, however, obtained; the saline was imperfectly absorbed from the peritoneal cavity in the animals with artificially raised blood ureas. The normal rabbit can remove 50 c.c. of saline completely from its peritoneal cavity within a few minutes, but in these animals the peritoneal cavities contained much free fluid half an hour to an hour after the injection of the saline. The free fluid was not accurately measured, but certainly

represented the greater part of the saline injected. The renal extraction ratios were quite normal in all these experiments, and the results have been recorded in the same table as those from animals which received no saline. The only recognizable effect of the intraperitoneal injection was that the operation was slightly impeded, as it was necessary to swab the free fluid out of the left renal pouch before needling the vein.

## CHEMICAL METHODS.

### CREATININE.

#### *Blood and urine.*

The creatinine was estimated by the ordinary method of Folin and Wu [1919]; *i.e.* tungstate protein-precipitations (in the case of blood) and alkaline picrate treatment of the filtrate for colorimetric measurement.

The only points requiring mention are: (1) the picric acid was recrystallized from boiling benzene and answered Benedict's test [1929] quite satisfactorily; (2) the creatinine content of the blood in the present investigations was always high—usually from 25 to 200 mg. per 100 c.c.

The method of estimation gives reasonably constant though rather low results. Estimations of creatinine added to blood in quantities varying from 10 to 200 mg. per 100 c.c. were between 93 and 94 p.c. of the actual at all levels.

Colorimetric readings are very easy at these high levels; an accuracy of  $\pm 1$  p.c. is not difficult even with the standard differing widely in strength from the solution under examination. In all the short sample experiments the renal vein blood was read directly against the heart blood as well as both against a standard; the results always showed close agreement.

#### *Kidneys.*

The creatinine was estimated in aqueous extracts of the kidneys, made by grinding up the kidney to a thin cream and extracting with about twelve volumes of water for 2 hours in the cold. The suspension was precipitated with tungstic acid in the same way as the diluted blood of the ordinary blood method. On addition of the alkaline picrate to the filtrate there was a sudden development of a greenish tinge, but this was very transient and had no influence whatever on the subsequent colorimetric readings.

Creatinine added to the original kidney paste was estimated subsequently to about 94–95 p.c. of the actual over a range of from 1 to



20 mg. per kidney. When the aqueous extraction was performed with boiling water only about 91 p.c. could be found in the filtrate.

All the figures for the creatinine in kidney and blood given in this paper are thus only about 94 p.c. of the true amounts: no correction has been made for this error, as it is constant throughout and does not effect any of the renal extraction ratios at all.

#### UREA.

##### *Blood.*

The method employed will not be gone into in great detail here, as it is to form the subject of another communication. It is a modification of Marshall's urease method in which the sources of error have been eliminated to a very considerable extent. It estimates only 94-96 p.c. of the absolute amount of urea, but the relative accuracy is quite good. Paired estimations, each on 2 c.c. of blood, were conducted on every blood sample, and in over three-quarters of the cases agreed to within 0.3 mg. per 100 c.c. As the blood ureas in the present work varied from 48 to 326 mg. per 100 c.c., and were usually between 100 and 200 mg. per 100 c.c., this agreement may be considered satisfactory. The results of three short sample experiments have been discarded as the agreement between pairs in one or other sample was unsatisfactory; in these cases the renal extraction ratio calculated from the lower renal vein blood determination and the higher heart blood figure was over 2 p.c. more than the ratio calculated from the higher renal vein figure and the lower heart figure. In the great majority of the experiments recorded, any slight differences in agreement between pairs were insufficient to affect the renal extraction ratio at all.

In the short sample experiments the renal vein and heart bloods were always set up in the same battery of apparatus for the estimation of urea, and the treatment of them was thus identical. Relatively to each other the figures are therefore reliable.

##### *Kidneys.*

The urea in the kidneys was estimated by the method of Marshall and Davis [1914]. Each kidney was extracted with 100 c.c. boiling alcohol, 2 c.c. of the solution thus obtained were evaporated to dryness in the reaction tube of the blood urea apparatus, the dry residue was redissolved in buffer phosphate solution in the tube, and the estimation continued as for blood urea. The method appears to give almost complete

extraction. As in the case of blood, only about 94–96 p.c. of the urea extracted is estimated in the subsequent chemical analysis, but this error is not of significance as it affects all estimations (both of blood and kidney) equally and so does not influence the renal extraction ratios at all.

#### SUMMARY.

1. After the intravenous injection of creatinine or urea into rabbits, comparisons have been made of the amounts of these substances reaching the kidney in the arterial blood and leaving it in the venous blood. The experiments were performed at various times, from a few seconds to  $3\frac{1}{2}$  hours after the injection, and at varying concentrations of the substances in the blood from about 20 to 200 mg. per 100 c.c. The percentage of the substance taken up by the kidney from the plasma passing through it is termed here the plasma renal extraction ratio.

2. During the first minute after the injection the mean plasma renal extraction ratio for creatinine is 31 p.c.; for urea it is 40 p.c.

3. During the next 20 min. the ratio for creatinine averages 19 p.c.; for urea it averages 2 p.c., and is sometimes negative.

4. Later than 50 min. after the injection the mean ratio for creatinine at low plasma concentrations is 38 p.c.; that for urea at low plasma concentrations is 15 p.c.

5. With increasing concentration of either substance in the plasma, a diminishing proportion is removed by the kidney from the blood. It appears probable that the absolute amount of these substances that the kidney can remove from the blood passing through it is limited by a maximum value of about 20 mg. of creatinine or 15 mg. of urea per 100 c.c. blood.

6. Combined experiments indicate that, when the kidney is removing very large amounts of creatinine from the blood, it removes little or no urea from the blood. When it is removing fairly large amounts of urea from the blood it can, however, still remove its normal amount of creatinine from the blood.

7. To explain all the results by a pure "filtration" theory, it is necessary to postulate the filtration of a third to a half of the plasma in the glomeruli, and the reabsorption of various amounts of urea and creatinine by the tubular epithelium. According to a pure "secretion" theory, the tubular epithelium must absorb various amounts of the creatinine or urea in the plasma directly from the intertubular capillaries; under certain circumstances up to about half. Either theory, or a com-

mination of both, may be correct as no fundamental impossibility is involved in any of the above explanations.

8. Nevertheless, it is definitely established here that under certain conditions a secondary return of urea from the renal parenchyma to the blood does occur.

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## THE CONTROL OF THE INSULIN OUTPUT OF THE PANCREAS.

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ALTHOUGH there is abundant evidence that the output of insulin from the pancreas is regulated according to the rate at which sugar enters the blood, yet opinion is divided as to the means by which this regulation is brought about. Some [Corral, 1918; McCormick, Macleod and O'Brien, 1923; Britton, 1925; Ahlgren, 1926; Clark, 1925, 1926, 1927, 1931; Debois, 1930; Zunz and La Barre, 1927, 1928; La Barre, 1928, etc.] regard the pancreas as being under nervous control by the vagus, though they differ among themselves as to whether the vagi (or one of them, and, if so, which one) provoke or inhibit the liberation of insulin, and also as to whether the response is a reflex, or is due to a direct effect of raised blood sugar on the nerve centres.

The alternative would be to consider the stimulus as a hormonal one, the pancreas directly responding to a raised arterial blood sugar by secretion of insulin. There is indeed evidence, *e.g.* that from experiments on denervation or transplantation of the pancreas, etc., which would seem unanswerably to support such a view [Banting and Gairns, 1924; Gayet and Guillaumie, 1927, 1928; Houssay, Lewis and Foglia, 1929; Wulf, 1929; Colwell, 1930, etc.].

An obvious experiment would be that of injecting glucose solution into the pancreaticoduodenal artery, by which the blood sugar would be raised considerably in the vessels of the pancreas but relatively little elsewhere. Such experiments have been carried out, and positive results claimed, by Grafe and Meythaler [1927, 1928], but these have been objected to by Geiger [1927, 1928] on the ground that the solutions of sugar used were grossly hypertonic. Further, both Grafe and Meythaler and Geiger used anaesthetized animals and thus introduced a complication. Accordingly the experiments about to be described were carried out mostly on decapitated cats, thus at once eliminating all possibility of central vagal control and of interference by anaesthetic.

## METHODS.

Fourteen experiments were completed. The cats were fasted for 20-45 hours, and were then decapitated under ether by Sherrington's method, or (in two experiments) anaesthetized by chloralose. Carotid blood samples were taken at intervals for estimation by the Hagedorn-Jensen method. After the first or second sample the abdomen was opened and the arteries for infusion dissected free; this caused no significant change in the blood-sugar curve, which was spontaneously falling following the decapitatorial rise. The sugar solution used for infusion was 5 p.c. glucose in saline or tap water, except in one experiment where 40 p.c. glucose in water was used. The slow infusion was made by the use of Burn and Dalé's apparatus [1924], either into the pancreatico-duodenal artery, or, for control, into the portal vein or femoral artery. The solution was introduced into the chosen vessel by means of the finest hypodermic injection needle, without any ligation of the vessel; so that the solution mixed with the blood flowing normally and uninterruptedly along the vessel. The greatest care was taken to give uniform and accurately known rates of infusion and, in the later experiments, the rate of flow was regulated by an assistant with a stop-watch who gave his attention solely to this task. At the end of all the later experiments some Chicago blue solution was passed in by the needle into the pancreatico-duodenal artery in order to ascertain the area of its distribution. This was found to be the whole head and neck of the pancreas, in all about half the organ. Carotid blood-pressures were observed at intervals, or at beginning and end of the experiments.

## RESULTS.

Control injections into the femoral artery appeared to give the same results as those into the portal vein.

As a result of the infusion of the sugar solution into the pancreatic vessels there was, as an immediate result in seven experiments, a rise of general arterial blood sugar, while in the other seven instances a slight fall occurred. In contrast with this, when the control injection was made into the portal vein or femoral artery (with only one exception), a rise of blood sugar always resulted.

Following either infusion there was in all but three instances (two following infusions into the leg, one into the liver) a fall of blood sugar, but the fall which always followed infusion with the pancreas was in

eleven experiments steeper than that following the control infusions; in the remaining three no controls or observations of fall were made.

The following protocols, one in which the infusion into the portal vein preceded that into the pancreas, the other in which infusion into the pancreas preceded the control into the femoral artery, are given by way of illustration.

*Exp. 7. 10. iv. 33. Cat (m.), 2.7 kg., fasted 45 hr. Glucose solution, 5 p.c. (in saline), infused into (1) liver through splenic vein, (2) pancreas through pancreatico-duodenal artery. Rectal temperature 36.5° C. throughout.*

Min.	Procedure	Blood glucose mg./100 c.c.	Rate of infusion c.c./10 min.	Arterial blood- pressure mm.
0	Ether	—	—	—
25	Decapitation	—	—	—
35	—	243	—	64
50	Operation	240	—	64
95	Infusion into portal vein	209	0	60
100	"	225	3	58
115	Infusion stopped "	243	3	54
125	—	224	—	—
150	Infusion into pancreas	215	0	55
167	"	228	3	—
180	Infusion stopped "	237	3	45
200	—	177	—	45
215	—	155	—	50

Distribution of pancreatico-duodenal supply shown to include head and neck of pancreas.

*Exp. 10. 25. iv. 33. Cat (m.), 3 kg., fasted 44 hr. Infusion of glucose solution, 5 p.c. (in saline), made as accurately as possible in rate, by employing a small burette. Both infusions were made in exactly the same way at a rate of 0.025 c.c. per 5 sec., by help of an assistant. Rectal temperature 39.5° C. throughout.*

Min.	Procedure	Blood glucose mg./100 c.c.	Rate of infusion c.c./10 min.	Arterial blood- pressure mm.
0	Ether	—	—	—
30	Decapitation	—	—	—
45	—	251	—	60
75	Operation	209	—	61
100	—	177	—	—
135	Infusion into pancreas	159	0	—
145	"	152	3	—
150	Infusion stopped "	166	3	—
160	—	136	—	—
170	—	124	—	—
185	—	113	—	—
195	Infusion into femoral artery	108	0	—
205	"	141	3	—
210	Infusion stopped "	132	3	—
220	—	125	—	—
230	—	110	—	—
245	—	101	—	45

Distribution of pancreatico-duodenal artery to head and neck of pancreas.

Although the blood-sugar level rose during both infusions, its extent in the pancreas infusion was smaller than that of the control, notwithstanding that the former was made for a longer period. The rate of increase during pancreas infusion was 7.3 mg./100 c.c. in 10 min. (22 mg./100 c.c. in 30 min.), whereas that in control infusion was 17.0 mg./100 c.c. in 10 min. (34 mg./100 c.c. in 20 min.).

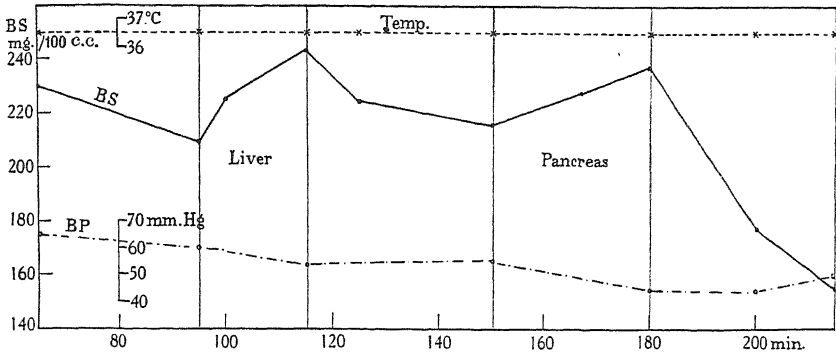


Fig. 1. Effect on blood sugar of decapitated cat of infusion of 5 p.c. glucose (0.3 g. in all) during 20 min. into portal vein and of 0.45 g. during 30 min. into pancreatico-duodenal artery.

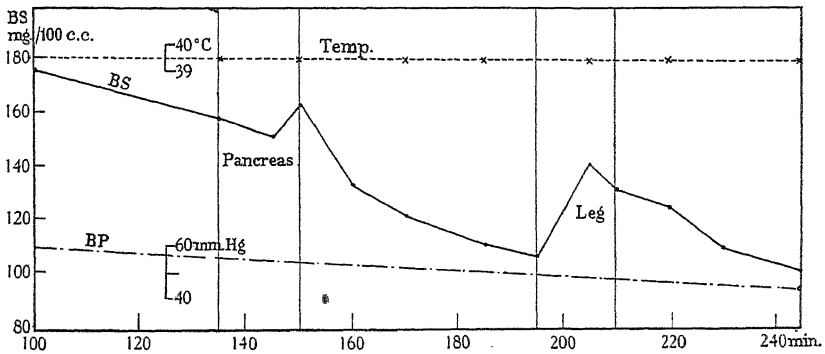


Fig. 2. Effect on blood sugar of decapitated cat of infusion of 5 p.c. glucose (0.225 g.) during 15 min. into pancreatico-duodenal artery and then (0.225 g.) during 15 min. into femoral artery.

On the contrary, the rate of fall in the blood sugar after the pancreas infusion was faster than that in the control, viz. 82 mg./100 c.c. in 35 min., as compared with 28 mg./100 c.c. in 35 min. The results are shown graphically in Fig. 1.

During the pancreas infusion there was, during the first 10 min., a fall of 9 mg./100 c.c., while in the leg infusion there was a rise of 33 mg./100 c.c. In the next 5 min. the blood sugar was increased slightly during the pancreas infusion. But the final rise of the blood sugar in the pancreas infusion was very small as compared with the control.

On the contrary, a more rapid fall in blood sugar was observed after the pancreas infusion than after the control, viz. 53 mg./100 c.c. fall in

TABLE I.

Exp. No.	Infusion into	Duration of infusion min.	Glucose infused g.	Rise in blood sugar produced mg./100 c.c.	Rate of rise (+) or fall (-) in blood sugar per 10 min. immediately following infusion mg./100 c.c.
1	Femoral artery	50	0.445	60	- 9
2	Femoral artery	55	0.415	- 101	—
	Pancreas	60	0.450	- 62	—
3	Femoral artery	10	0.15	12	- 14
	Pancreas	10	0.15	- 29	- 18
	Femoral artery	10	0.10	3	+ 24
	Pancreas	30	0.325	- 43	—
4	Femoral artery	15	0.15	35	+ 31
	Pancreas	28	0.42	- 59	- 13
5	Portal vein	25	0.375	21	- 17
	Pancreas	50	0.60	- 33	- 23
6	Portal vein	20	0.30	40	+ 3
	Pancreas	30	0.45	- 12	- 4
	Pancreas	30	0.50	5	- 9
7	Portal vein	20	0.30	34	- 19
	Pancreas	30	0.45	22	- 30
8	Portal vein	25	0.375	44	- 4
	Pancreas	25	0.375	27	- 14
9	Pancreas	30	0.45	- 2	- 33
	Portal vein	30	0.45	65	- 12
10	Pancreas	15	0.225	7	- 30
	Femoral artery	15	0.225	24	- 7
11	Pancreas	15	0.225	36	- 38
	Femoral artery	15	0.225	9	- 32
12*	Pancreas	15	0.225	6	- 14
	Femoral artery	15	0.225	14	- 7
13†	Pancreas	5	0.6	50	- 32
	Pancreas	5	0.6	59	- 32
	Pancreas	5	0.6	43	- 40
14‡	Pancreas	15	0.18	- 21	- 14
	Femoral artery	15	0.225	18	0
	Pancreas	15	0.225	12	- 4

\* Chloralose.

† Chloralosed animal. Injection of 40 p.c. glucose at rate of 0.3 c.c. per min.

‡ Cat given 0.1 g. thyroideum siccum daily for 16 days previously.



35 min. following the pancreas infusion, and 31 mg./100 c.c. fall in 35 min. following the control infusion. This experiment is shown graphically in Fig. 2.

Table I shows in summary the rates of rise during, and of fall (or rise) following, the various infusions. The last column shows the fall (or rise) in the first 10 min. after termination of the infusion, as observed, or, when no sample was taken at 10 min., as calculated.

These experiments seem not only to answer affirmatively the question as to whether the blood-sugar level directly affects the insulin output of the pancreas, but also to answer the objection raised by Geiger to the work of Grafe and Meythaler. The solution here employed was not hypertonic, and though the fall of blood sugar following pancreatic infusion was less dramatic than that obtained by Grafe and Meythaler, it was nevertheless clearly greater than that seen after the infusion of either liver or leg. Since the results obtained on decapitated cats were similar to those obtained with those under chloralose, it would seem that control from the higher centres plays no essential part in producing the response. This, of course, is not to say that the response is incapable of being modified by nervous action. Moreover, the liver itself appears to play no direct part in causing the effect, because the results of infusion into the portal vein are on the whole similar to those got by infusion into the femoral artery.

One observation, for which no evident explanation suggests itself, is the failure in many instances of the blood sugar to rise when an infusion was made into the pancreatic vessels, although a similar or even a smaller quantity of glucose, when injected into the femoral artery or portal vein, caused a rise of blood-sugar level.

#### SUMMARY.

Infusion of 5 p.c. glucose into the blood stream along the pancreaticoduodenal artery in decapitated cats, thus exposing about half the pancreatic tissue to a glucose concentration above the normal, is followed by a more rapid decline in arterial blood sugar than occurs when similar injections are made into the femoral artery or portal vein. It is concluded that the pancreas responds promptly to a high sugar content of its arterial blood by a liberation of insulin.

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I wish to express here my sincerest thanks for this, and to Prof. C. Lovatt Evans for suggesting the work, and for his help and encouragement throughout.

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## THE OUTLINE METHOD FOR INVESTIGATING GASTRIC MOTILITY.

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FOR the satisfactory study of the motility and rate of emptying of the stomach, it seems essential that the method used should involve the minimum of interference with that organ or its contents during the periods of observation. The ideal method should enable records to be taken of the intact stomach in its normal surroundings, and containing nothing but normal physiological contents or specific substances which might be under investigation. The methods in use at the present day possess certain inherent disadvantages as judged by this standard: by overcoming some of these difficulties, it is hoped that the "outline method" described in this paper will provide a useful addition to the technical procedures employed in the study of gastric motility.

The utilization of gastric and duodenal fistulæ has been a recognized procedure ever since the classical case of Alexis St Martin. An animal with a gastric fistula can, however, never be considered to possess a healthy stomach: great care must therefore be taken in the evaluation of results obtained thereby. Carlson [1912] has shown clearly that the hunger contractions demonstrated by Boldireff [1905] in his dogs with fistulæ correspond in type to those observed by Carlson in old or debilitated animals in which the contractions were recorded by means of a balloon in the intact stomach. Clinically it is quite apparent that gastrostomies never satisfactorily replace oral feeding.

The utilization of gastric pouches merits the same criticism. Carlson [1912] investigated the relationship between the movements of a Pavlov pouch and those of the reconstituted stomach; he found the relationship extremely variable and apparently dependent upon the size and condition of the bridge of tissue between the two parts. Robins [1923] could detect no correlation between the movements of a Heidenhain pouch and the parent stomach. Still less certain will be the conclusions

drawn from the movements of a transplanted pouch where all muscular and nervous connections are completely divided [Ivy and Farrell, 1925].

The opaque meal possesses the great advantage of utilizing the intact stomach in its normal surroundings. Its inherent defect lies in the necessity of introducing a mass of foreign material which exerts an unknown influence upon the motor response of the stomach. It forms an admirable comparative method, whereby stomachs can be contrasted in terms of their reaction to a standard opaque meal, and it enables a detailed study of contractions and emptying rates to be made under these conditions. The method has, however, definite limitations, as it does not permit of the study of the empty viscus nor the investigation of the response of the stomach to any particular foodstuff or substance, as the presence of the opaque material may conceivably modify the result obtained.

The fractional test-meal can supply indirect information as to motility: the emptying time for any particular constituent of the meal can be determined, and the secretory curves give a measure of the time taken by the stomach to return to its resting state. It provides, however, no information as to the dimensional changes of the organ during the meal, unless the procedure of periodical withdrawal and return of the gastric contents is adopted: this seems a highly unphysiological procedure, as the well-recognized rapid adaptation of the stomach to variations in the volume of its contents would necessitate a gymnastic display quite foreign to the orderly procedure of the uninterrupted passage of a meal. The tube in the stomach is in itself a drawback owing to the great individual variations in the tolerance of its presence.

The object of the "outline method" is the provision of an animal in which the outline of the stomach can be rendered visible by X-rays without in any way interfering with the contents of the organ. A series of fine silver wire sutures are attached to the serous coat along the optical margins of the stomach; X-ray photographs are then taken, and the line of sutures enables the construction of an outlined diagram of the stomach at any phase of its activity.

#### THE METHOD.

Apart from a few preliminary trials on cats, dogs have been used throughout the work. Some care must be exercised in the selection of the animal: it should be amenable to training, and a preliminary trial in the X-ray room should be carried out. A medium-sized terrier with a broad chest and wide subcostal angle is the most suitable—the latter

point is of importance, as it increases the ease of access to the cardiac region when the sutures are being applied. After trial of several types of material, *e.g.* Michel clips and fine silver clips of various shapes, the most suitable medium was found to be fine silver wire of 0.17 mm. diameter: this can be threaded on fine cleft-palate needles and manipulated with the same ease as stout thread.

The object of the method is to define the outline of the stomach by the application of a series of opaque sutures along the optical margins of the organ. The optical margins of the stomach will vary according to the position employed for photography and with the degree of filling of the organ. The first of these variations can be eliminated by the adoption of a standard position for photography, and we have used a dorso-ventral exposure of the animal standing in its normal erect position. For this standard exposure we have determined the anatomical landmarks which correspond to the optical outline of the stomach, and have applied our sutures accordingly. If the sutures are faithfully applied to the anatomical lesser and greater curvatures, it is found that the suture line accurately depicts the outline of the stomach for the whole of the lesser curvature and the pyloric half of the greater curvature. In the region of the body, however, it is found that the line of sutures falls well within the borders of the fundic shadow, so that in this region the anatomical and optical curvatures do not coincide. Dogs with sutures applied along the anatomical curvatures always showed a marked optical defect in the region of the fundus (Figs. 1, 2), and it was necessary to modify the disposition of the sutures in this region in order to perfect the optical outline. In actual fact, the anatomical greater curvature passes on to the dorsal aspect of the fundus of the stomach, and the optical margin must therefore lie anterior to the anatomical boundary. The following procedure has been adopted for the satisfactory definition of the optical greater curvature: starting at the pylorus, the anatomical greater curvature is followed until the lower pole of the spleen is reached, and from this point the suture line is made to diverge from the anatomical curvature, the sutures being inserted along the anterior surface of the stomach till they reach a point  $\frac{3}{4}$  in. internal to the line of reflection of the gastro-splenic ligament at the level of the upper pole of the spleen. At this point the suture line stops, for experience has shown that it is quite unnecessary to attempt to outline the fundic cap, the shadow of which is always clearly visible owing to its contents. It should be noted that the sutures are applied under ether anæsthesia; in this condition the stomach is generally very atonic, and thus gives a wrong impression

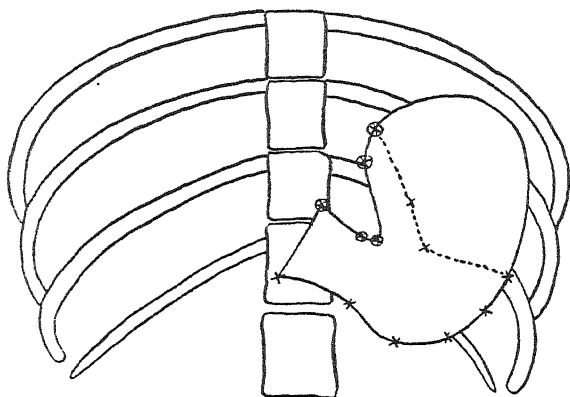


Fig. 1. Fasting stomach of Dog G 5. ⊗ Sutures on lesser curvature. × Sutures on greater curvature. In this dog, the sutures had been placed along the anatomical curvature; hence the marked defect in the outline of the fundus as shown by the dotted line. The continuous outline of the fundus was obtained from the shadow of the fundic contents.

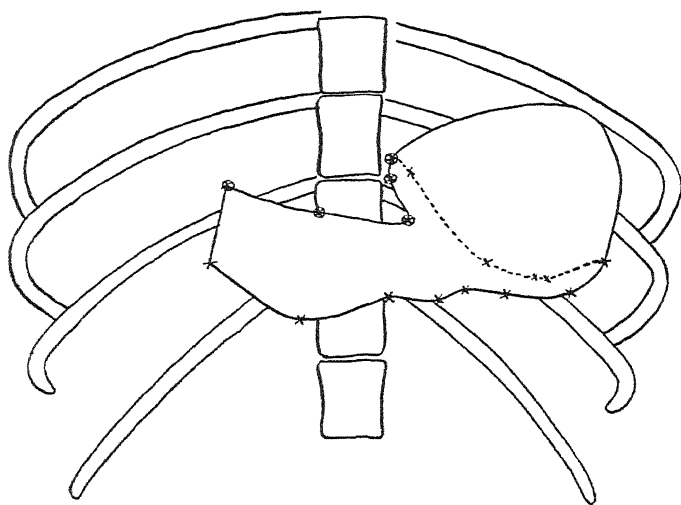


Fig. 2. Fasting stomach of Dog G 6. Tubular stomach suggesting poor tone. ⊗ Sutures on lesser curvature. × Sutures on greater curvature. These sutures were applied to the anatomical curvature giving similar defect as Fig. 1.

as to the width of the gap between the end of the suture line and the cardia. When the animal recovers and the fasting stomach is X-rayed, one is surprised to find how closely the suture line approaches to the cardia (Fig. 6).

#### TECHNIQUE.

Under ether anæsthesia, the abdomen is opened through a left paramedian incision, and the stomach is picked up in sponge rubber forceps which allow of the necessary manipulations with the minimum of trauma. Starting at the cardia, a series of fine silver sutures is applied along the lesser curvature at the point of attachment of the anterior layer of the lesser omentum. When the pylorus is reached, attention is directed to the greater curvature, and the sutures are applied along the optical outline as previously described. The sutures are applied under direct observation, and great care is exercised to avoid vessels and nerves. Each suture just penetrates the serous coat, and is tied in a loose loop to avoid strangulation of the included tissue. The number of sutures employed depends upon the size of the stomach and the particular object of the preparation; for the general purpose of determining the whole gastric outline, the sutures are applied at intervals of  $\frac{1}{2}$  in., so that about eight are required for the lesser curvature, and about fifteen for the greater. The stomach is then inspected for bleeding points and loose or projecting pieces of wire, the abdomen is closed in layers, and a bandage applied.

After the operation, the animal is returned to a cage in a warm room, and morphia injected to keep it quiet for the ensuing 24 hours. A fluid diet is given for the following 2 days, and the animal then returns to its own cage and a normal diet. Adopting this procedure, we have had no trouble with the healing of wounds, and the animal's appetite after the first days seems quite unaffected by the manipulations.

The subsequent history of animals so treated suggests that they suffer no disability or discomfort from the presence of the sutures. Their appetites, weight and general activities are unimpaired. Of the six animals in which this method has been used, two are alive and well after intervals of 15 and 12 months respectively. Dog G 5 was killed at the end of 18 months, Dog G 6 at the end of 23 months, and Dog G 8 at the end of 4 months, in order to investigate the condition of the stomach. Dog G 7 died of suppurative hepatitis 2 months after the operation. Post-mortem examination of these stomachs showed the sutures in position with no evidence of any local damage to the muscle or mucosa:

there were little patches of peritoneal thickening round each piece of metal, but no adhesions or deformity of the stomach could be seen. From these observations it seems justifiable to conclude that the presence of these sutures in the serous coat produces no significant effect upon the stomach.

#### METHOD OF RECORDING.

In order that the photographic records shall be strictly comparable, it is essential to establish standard conditions of exposure, and for each animal there must be a fixed relationship between the position of the tube, animal and film. To ensure this uniformity of conditions, the animals

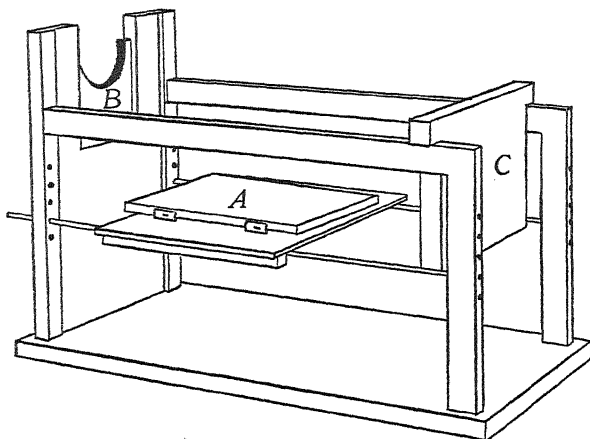


Fig. 3. X-ray stand for dog. A. Film cassette on adjustable platform. B. Adjustable neck-piece. C. Moveable tail-piece.

are taught to stand in a wooden frame which ensures an erect attitude without undue restriction: a centring device correlates the position of the tube and film relative to a fixed point on the animal's spine.

The frame consists of a firm rectangular wooden base with wooden uprights at each corner. The two front uprights are united by a slatted board with a curved neck-piece which can be adjusted to the height of the dog. On each side the uprights are connected by top-rails which prevent the animal lolling to one side, but allow it to stand freely in the erect attitude. The back of the frame is left empty to allow the animal to enter; when the animal is in the frame, with its head projecting over the neck-piece, a tail-piece is adjusted behind so that the animal is enclosed on all sides (Fig. 3). The frame is essential to ensure a uniform



posture, but its dimensions are such that it offers no restriction provided the animal stands erect.

When the animal is in position, a wooden platform carrying the film cassette is slid into position under the dog's abdomen—the height of this platform is adjustable, its setting being fixed for each dog to ensure uniform relationship between the animal and film. The tube is set at a standard height of 24 in. above the film, and by means of a plumb-bob it is centred over the dog and film. To aid this centring, a lead bullet is fixed with collodion in the middle-line of the dog's back over the first lumbar vertebra, and the film cassette has two pairs of cross-wires which, by their intersection, outline a square centimetre over the centre of the film. With very little practice, the dog can be taught to stand in the frame, and the plumb-bob of the tube is aligned with the lead shot and wires in the transverse and longitudinal axis of the dog. The exposure is therefore made under strictly standard conditions. The X-ray film shows the position of the cross-wires and lead shot, and if centring has been satisfactory, the shot should lie in the centre of the square over the first lumbar vertebra.

Using a 30 M.A. fine focus Coolidge tube, 98 k.v., 60 M.A., exposures of  $1/20$ – $1/10$  sec. were found sufficient.

With this technique it is possible to make photographic studies of the empty stomach and to record the changes in outline during the passage of a meal. Tracings are taken from the films, and the gastric outline is constructed from the line of sutures and the fundic shadow.

In order to obtain some simple quantitative measurement which would serve as an index of the size of the gastric outline, measurements have been made of the length of the lesser and greater curvatures. The length of the lesser curvature shows very little alteration for any phase of gastric filling, and cannot be correlated with changes occurring in the dimensions of the stomach (Fig. 4). The greater curvature, however, shows very definite alterations in length, and this measurement can be utilized for studying the rate of emptying of the organ. If the length of the greater curvature is plotted against the time intervals following the ingestion of a meal, a curve is obtained which shows a steady diminution in the length of the curvature until a stationary level is reached—the time at which the curve flattens out corresponds with the time of emptying of the stomach (Fig. 5). This method of measurement has been employed in all the experiments on the rate of emptying of meals, the curves providing a simple and rapid method of comparison.

It might be objected that the measurement of one selected portion

of the gastric outline provides an unfair index of the dimensional changes of the organ. It must be clearly understood that no attempt is made to establish any quantitative relationship between the length of the greater curvature at any moment and the corresponding gastric volume beyond

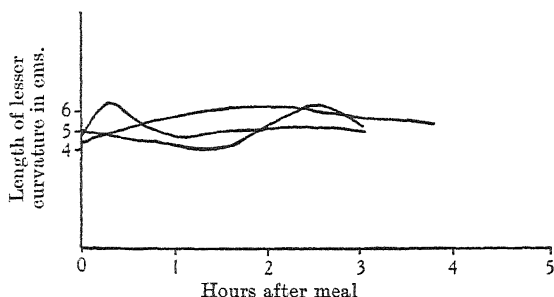


Fig. 4. Length of lesser curvature after 350 c.c. milk. Three similar meals on G 5. Note the uniformity in length at all stages after meal.

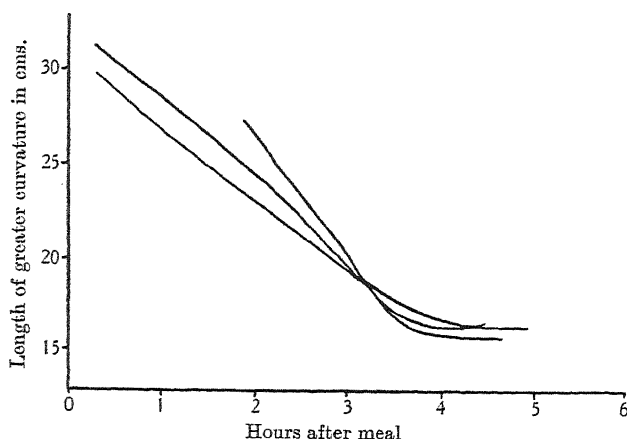


Fig. 5. Length of greater curvature after 350 c.c. milk plus 1 g. NaCl. Three similar meals on G 5. Note the steady diminution in length till the curves suddenly flatten out at the emptying point of  $3\frac{3}{4}$ –4 hours.

the assumption that, in the same animal, similar values of the length of the greater curvature indicate approximately similar gastric dimensions. Inasmuch as the stomach is a muscular tube, the variations in the capacity of which are made mainly at the expense of the greater curvature, it would appear quite reasonable to use the latter measurement as an index of changes occurring in the former.

## THE FASTING STOMACH.

Studies have been made of the gastric outlines of dogs which have been deprived of solid and fluid food for 24 hours. This procedure is a routine preliminary to all the experiments we have made. For the stomach under such conditions the usual term "empty" is a misnomer, as some gastric contents are invariably present: consequently, we have adopted the term "fasting" as being more accurately descriptive.

The two dogs employed in the earlier observations by this method present very different forms of gastric outline. Dog G 5, a lively wire-haired terrier, has a definitely J-shaped stomach (Fig. 1), whereas the stomach of Dog G 6, a placid, lethargic Irish terrier, appears as a tubular structure slung transversely across the abdomen (Fig. 2). As these photographs are all dorso-ventral exposures, the presence of a definite J-shape indicates good muscle tone, whereas the simple transverse tubular organ suggests a hypotonic viscus. These differences can be paralleled in the case of the human stomach, bearing in mind the difference in attitude between biped and quadruped. The long dependent J-shaped stomach, typical of hypotonia in man, corresponds with the transverse tube of Dog G 6, whilst the "steer-horn" stomach corresponds to the J-shaped stomach of Dog G 5. The general outline shows clearly that the fasting stomach is not a sac in which the contents collect in the most dependent parts: it is a tube with walls in sufficient tone to maintain the resting contents in the fundic portion, and closely corresponds with the form described by Stopford [1913] for the human stomach.

## WAVES OF CONTRACTION.

It has been possible to demonstrate the presence of waves of contraction in the fasting stomach, and with experience a series of exposures at 15-30 sec. intervals can be made with the animal remaining motionless in the frame. A comparison of the outlines of such a series of photographs demonstrates the presence of waves of contraction, and an approximate estimate of their periodicity and duration is possible. Attempts to modify the activity of the empty stomach by disturbing sounds or savoury smells produced no definite results.

## INFLUENCE OF EXTERNAL TEMPERATURE UPON GASTRIC TONE.

After numerous observations had been made on these dogs under uniform conditions, the dimensions of the outline of the fasting stomach were well established and were remarkably constant for each animal.

Quite suddenly it was noticed that there was a very definite increase in the size of the fasting stomach: this was so definite that the animal attendant was accused of feeding the animals during the period when they should have been deprived of food. In a search for the cause of this sudden alteration, it was noted that the change coincided with the installation of thermostatically controlled electric radiators in the animal house. Previously the rooms were heated by hot water pipes, and during the winter the animals were subjected to considerable fluctuations between day and night temperature. As soon as the heating was stabilized, the animals became more peaceful, and at the same time the dimensions of their fasting stomachs increased. In order to test the connection between the two phenomena, the electric heating was discontinued, and it was found that when the minimum nocturnal temperature fell to about 45° F., the original small stomach was obtained. The change to the uniform electrical heating produced a 17 p.c. increase in the length of the greater curvature of Dog G 5, and an increase of 23 p.c. in Dog G 6. This variation in the dimensions of the fasting stomach suggests an alteration of gastric tone as part of the reaction of the animal to fluctuations in external temperature.

#### THE PASSAGE OF A MEAL THROUGH THE STOMACH.

As a basis for comparing the rate of emptying of various foodstuffs, a meal of skim milk has been used as a standard, and the emptying time determined for each dog. The volume of the meal is adjusted to the size of the animal—small dogs receiving 250–350 c.c., large dogs, 500 c.c. The milk is given slightly warmed, and the animal drinks it readily. Skim milk has been chosen as being practically fat-free, isotonic with blood and readily taken by the animals. The routine procedure adopted is as follows. The animal is deprived of food for 24 hours, a photograph of the fasting stomach is taken, and then the meal is given. A photograph of the full stomach is taken and repeated at intervals of an hour until the stomach approaches its fasting dimensions, when the exposures are made at intervals of half an hour. When two consecutive photographs show that the stomach has reached its stationary fasting level, the meal has left the stomach.

A comparison of the outline and distribution of sutures in the fasting and full stomach shows very clearly that the main increase in gastric capacity is made at the expense of the greater curvature of the stomach, particularly in the fundus and proximal part of the body. In the fasting

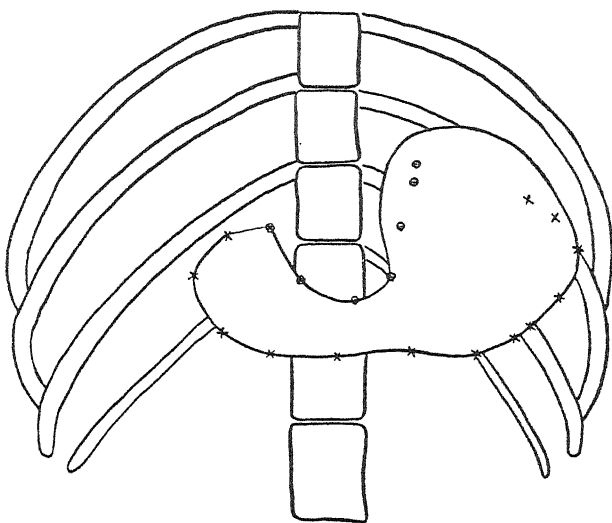


Fig. 6. Fasting stomach of Dog G 9. Eight sutures on lesser curvature, thirteen sutures on greater curvature. These sutures were applied to the "optical" curvatures (see text) with consequent improvement in outline.

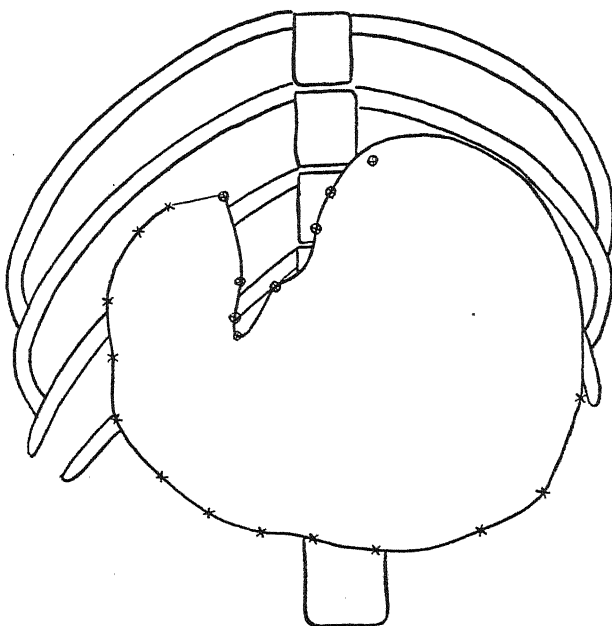


Fig. 7. Stomach of Dog G 9 1 hour after milk meal. Eight sutures on lesser curvature, thirteen sutures on greater curvature. Note the increase in interval between the cardiac sutures of each curvature.

stomach the top suture on the greater curvature lies very close to the cardiac suture on the lesser curvature (Fig. 6): in the full stomach the interval between these two sutures is very much increased, as is also the interval between individual sutures on the proximal half of the greater curvature (Fig. 7). As the lesser curvature appears to vary very little, this alteration in the relative position of the sutures must indicate a marked change in the dimensions of the fundic portion of the greater curvature. This finding is in keeping with the morphological difference between the two portions of the stomach. The lesser curvature is related to the primitive digestive tube, which persists as such in ruminants and kangaroos, and appears to be associated with the conduction of fluid. Accordingly, it is not surprising to find no great variations in the linear dimensions of the lesser curvature, as it is concerned with conduction and not storage. The greater curvature represents the outline of the cæcum-like diverticulum developed from this primitive digestive tube for the purpose of accommodating food; as such, it must be capable of adaptation to alterations in the volume of its contents and, according to the degree of filling, variations in the dimensions of its outline are to be expected.

It is realized that an alteration of the gastric axis could, in itself, alter the apparent relationship of the sutures as seen in the one plane of vision employed. Although such an alteration may take place, we do not think it is responsible for any very marked change in gastric outline, as the dimensions of the lesser curvature remain so constant and it is very unlikely that such an axial movement could affect one curvature only. Furthermore, the relationship of the sutures to the actual shadow of the greater curvature (which is frequently visible) remains constant.

We have taken as the emptying time for any meal the period elapsing between the taking of the meal and the time at which the gastric dimensions assume a stationary level comparable to that of the fasting stomach, *i.e.* the point at which the curve of greater curvature length flattens out (Fig. 5). This assumption of the stationary condition suggests that the stomach has returned to its resting state following the passage of all the food into the duodenum, but it is necessary to prove this before accepting the above as criteria of "emptying." To test this, a trace of barium is added to the standard meal so that the escape of material from the stomach can be directly observed—the record of such "light" barium meals shows that the point of emptying of the meal does coincide with the assumption of a stationary level of gastric dimensions: therefore we

feel justified in adopting the above criteria as a basis for the comparison of "emptying times."

Adopting this standard of measurement, repeated observations have been made on the dogs; the emptying times for each animal have been remarkably constant, though wide variations occur between individual animals.

TABLE I.

Dog	Meal	Emptying time hrs.
G 5	350 c.c. skim milk	3
		$3\frac{1}{4}$
		$3\frac{1}{2}$
		3
G 9	250 c.c. skim milk	$3\frac{1}{2}$
		$3\frac{3}{4}$
	350 c.c. skim milk	$4\frac{1}{2}$
		$4\frac{1}{4}$
G 6	500 c.c. skim milk	$4\frac{1}{2}$
		$4\frac{1}{2}$
		5

In ordinary X-ray pictures one frequently sees shadows which might possibly indicate the outline of the stomach, but, owing to the uncertainty of their identification, very little reliance can be placed upon them. The presence of the suture-line immediately picks out those shadows related to the stomach, so that its outline can generally be mapped out with an accuracy not directly dependent upon the actual sutures—this fact serves as a very useful counter-check upon the position of the sutures, and with a little practice one soon learns the degree of accuracy with which the sutures have been placed in the individual dogs.

#### DISCUSSION.

The "outline method" for gastric investigation possesses certain definite advantages over the other methods available. It provides an animal in which the dimensions of the stomach can easily be followed without in any way disturbing that organ or its contents. It is therefore well adapted to the study of the emptying rate of the stomach for various foodstuffs or to the study of the influence of specific substances upon this rate. It enables one to assess the part played by the various portions of the stomach in accommodating the gastric contents and can demonstrate simultaneously segments which are filled or empty. It appears to be the only method available for studying the "fasting stomach" in its normal surroundings.

In use, it has proved to be a practicable method. Animals of this kind have been employed for over two years upon studies of the influence of osmotic pressure and fat content of meals. The presence of the sutures appears to exert no deleterious influence for periods of at least two years. With a little practice the photographs are easy to interpret, and the outlines can be rapidly drawn and measured.

The method appears capable of development in two directions. By a closer distribution of the sutures in any one segment of the stomach, a more detailed analysis of its activity should be possible: this is particularly true of the distal half of the stomach where a strictly accurate outline is most easily obtained. It should also be applicable to other hollow viscera, such as the bladder, gall bladder, uterus and parts of the colon where axial rotation is not prone to occur.

#### SUMMARY.

A method for studying the motility of the stomach is described. The method consists of applying interrupted fine silver wire sutures to the optical margins of the stomach. X-ray photographs of the gastric outline are taken at any desired phase of gastric activity.

Variations in size and waves of contraction of the fasting stomach have been studied in different animals. The size has been found to be affected by external temperature.

Dimensional changes of the stomach during the passage of a standard milk meal are described.

One of us, W. R. Spurrell, is indebted to the Medical Research Council for a personal grant.

The expenses of this investigation have been defrayed in part by a grant from the Government Grant Committee of the Royal Society.

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INFLUENCE OF OSMOTIC PRESSURE UPON THE  
EMPTYING TIME OF THE STOMACH.

BY B. A. McSWINEY AND W. R. SPURRELL.

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THE influence exerted by the osmotic pressure of the gastric contents upon the rate of emptying of the stomach has been investigated by numerous workers, and while all observers are in agreement that hypertonic solutions lead to delay in the emptying of the organ, considerable difference of opinion exists as to the effects of hypotonic solutions. These results have been obtained by the use of two types of experimental method: (a) the determination of the volume of the meal remaining in the stomach after an interval, (b) the measurement of the quantity expelled through a duodenal fistula.

Best and Cohnheim [1910] used dogs with a gastric fistula, and measured the volumes of gastric contents 10 minutes after the introduction of meals of various osmotic pressures. Macleod and others [1930], using rats, killed the animals with a blow on the head, rapidly isolated the stomach and intestine, and determined the proportion of meal present in those segments. These methods can give no data as to the total emptying time, and involve considerable interference with the normal physiological condition of the stomach. Powerful contractions of the stomach are frequently seen as an animal dies, and post-mortem findings as to the relative volume of gastric contents may be very misleading as a result of a few terminal gastric convulsions.

Carnot and Chassevant [1905] and Otto [1905] measured the outflow through duodenal fistulae: they obtained measurements of the total emptying time and studied the changes in the osmotic pressure of the escaping fluid. The modern view of the part played by duodenal absorption in regulating gastric motility introduces an element of uncertainty into results based on such fistula methods. The position of the fistula is usually just below the point of entry of the bile duct, so that the area available for duodenal absorption is very small and the contents are rapidly drained away through the fistula. It may be that the rate of

emptying under these conditions is mainly controlled by the conditions existing in the stomach itself with little or no modification from the duodenum.

Our attention was drawn to this question during an investigation of the properties of certain commercial "meat extracts" which enjoy a reputation as "gastric stimulants." In acute experiments upon the movements of the stomach, the use of these extracts had become a routine to ensure an active preparation. After establishing the emptying time for standard milk meals in dogs by the "outline method," commercial "meat extract" was added to the milk with the expectation that it would accelerate the emptying of the stomach, but on the contrary the meal produced marked delay. In a search for the explanation of this delay, our attention was drawn to the high salt content of these extracts, and experiments were performed to see whether the alteration in osmotic pressure resulting from the addition of the extract to the milk was in any way a contributing factor. The "outline method" [McSwiney and Spurrell, 1933] seemed very suitable for such investigations, as no disturbance of the normal relations of the stomach and duodenum is incurred.

#### METHOD.

*Hypertonic solutions.* The addition of commercial "meat extract" in the quantities employed altered the  $\Delta$  of the milk meal from  $0.55^{\circ}\text{C}$ . to  $0.91^{\circ}\text{C}$ . Meals of the same osmotic pressure were made up by the addition of NaCl or glucose to the milk. The addition of 2 g. NaCl or 11 g. glucose to 350 c.c. milk raised the  $\Delta$  of the mixture to  $0.9^{\circ}\text{C}$ ., and these meals were given to two adult dogs prepared for the "outline method." Meals with an intermediate osmotic pressure were also prepared by the addition of 1 g. NaCl or 5.5 g. glucose to 350 c.c. meal. All meals were duplicated in each dog. Table I gives a typical series of results for Dog G 5 and Fig. 1 gives a graphic record of the corresponding alterations in the length of the greater curvature.

TABLE I. Emptying time for meals of different osmotic pressure.

Dog G 5. Meal = 350 c.c.		
Meal	Depression freezing point of meal $^{\circ}\text{C}$ .	Emptying time hrs.
Milk	0.55	3-3½
Milk + glucose	0.712	4½
Milk + NaCl	0.723	4-4½
Milk + NaCl	0.905	4¾
Milk + meat extract	0.913	5
Milk + glucose	0.925	4¾

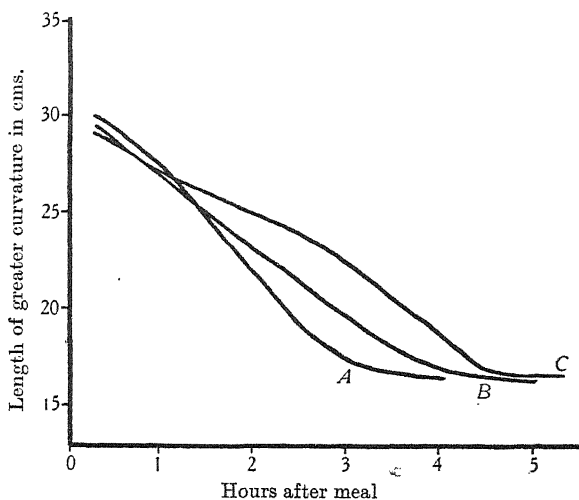


Fig. 1. Lengths of greater curvature after hypertonic meals: A. 350 c.c. of skim milk  $\Delta 0.55^{\circ}\text{C}$ . B. 350 c.c. of skim milk + glucose  $\Delta 0.712^{\circ}\text{C}$ . C. 350 c.c. of skim milk + glucose  $\Delta 0.925^{\circ}\text{C}$ .

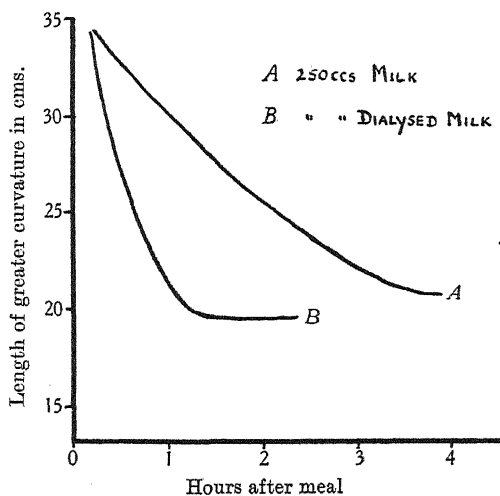


Fig. 2. Lengths of greater curvature after hypotonic meals: A. 250 c.c. of skim milk  $\Delta 0.55^{\circ}\text{C}$ . B. 250 c.c. of dialysed milk  $\Delta 0.035^{\circ}\text{C}$ .

*Hypotonic solutions.* Milk was dialysed against three changes of distilled water in 24 hours and the  $\Delta$  reduced to  $0.035^{\circ}\text{C}$ . Meals of 250 c.c. of this hypotonic fluid were given to two dogs, and the emptying time determined in duplicate (Fig. 2). The milk underwent a little dilution during dialysis, but the effect of this was checked by the control meals of milk diluted to an equal extent with isotonic saline. This degree of dilution was found to have no appreciable effect upon the emptying time.

TABLE II. Emptying times for hypotonic meals.

Meal = 250 c.c.

Dog	Skim milk hrs.	Dialysed milk hrs.
G 9	$3\frac{1}{2}$ – $3\frac{3}{4}$	$1\frac{1}{2}$ – $1\frac{1}{2}$
G 10	$3\frac{1}{2}$	$1\frac{1}{2}$ – $2\frac{1}{4}$

*Experimental results.* Hypertonic solutions produce delay in emptying proportional to the rise of osmotic pressure employed. As iso-osmotic mixtures of milk and commercial "meat extract," milk and NaCl, milk and glucose produce a similar degree of delay, it is clear that the delay is not due to any particular constituent of the meals but to the common factor of hypertonicity. Hypotonic solutions (as seen from Fig. 2) empty more rapidly than isotonic.

#### DISCUSSION.

All observers have recorded a delay in the emptying of hypertonic solutions, and the delay is proportional to the degree of hypertonicity: our results agree with these findings. Miller and others [1920] found the same degree of delay for test meals of hypertonic glucose as for hypertonic cane sugar, but Carnot and Chassevant [1905] considered that meals of hypertonic glucose produced less delay than saline of the same osmotic pressure. According to our observations, the degree of delay is not appreciably affected by the nature of the material employed to raise the osmotic pressures.

It is rather surprising to find relatively small changes in the osmotic pressure producing such marked delay. The addition of 2 g. NaCl to 350 c.c. skim milk is not far removed from the range of ordinary culinary procedure, so that quite wide variations in the emptying time of standard meals may be expected if strict attention is not paid to the salt content.

One is faced with considerable difficulty in assessing what is generally meant by the emptying time for a meal. If the meal contains one single constituent, the time of its disappearance from the stomach is obviously

the emptying time. Meals rarely consist of a single constituent, and it is not justifiable to select a particular component, follow its course through the stomach and apply the results obtained to the whole meal, *e.g.* the emptying point of a gruel meal is not necessarily the point of disappearance of carbohydrate from the stomach. It would appear more reliable to determine the time taken for the stomach to resume its resting state, *i.e.* to determine the period of gastric activity in response to a meal, and it is the measurement of this quantity that we have made and compared by the "outline method."

The dimensional changes undergone by the stomach as the result of a meal would appear to depend upon a balance between three factors:

- (1) Amount of material escaping into the duodenum.
- (2) The volume of material added to the gastric contents, *i.e.* gastric secretion, swallowed saliva and duodenal regurgitation.
- (3) The absorption of water by the stomach.

All observations on the rate of pyloric outflow have shown that this is diminished in the case of hypertonic solutions. Carnot and Chassevant [1905], Macleod and others [1930] found evidence of duodenal irritation as a result of the escape of such solutions, and it seems probable in their experiments that the pyloric mechanism was affected thereby. In addition, the presence of hypertonic fluids in the stomach gives rise to a copious secretion which dilutes the contents and greatly increases their volume, *e.g.* a 60 c.c. meal of meat extract in a cat will produce 40 c.c. of secretion in an hour, and 20 p.c. glucose in the rat's stomach is diluted 40–90 p.c. in 1 hour [Macleod and others, 1930]. The amount of water absorbed by the gastric mucosa is influenced by the osmotic pressure of the gastric contents, and hypertonic solutions will tend to diminish this process. Thus, all three factors will unite in maintaining the volume of the gastric contents and so producing a delay in emptying.

In respect of hypotonic solutions, Carnot and Chassevant [1905] state that they escape through the pylorus slightly more slowly than isotonic, though they give no details of measurement. Otto [1905] also makes the same statement, but graphs constructed from his protocols give exactly the same emptying time for iso- and hypotonic solutions. Best and Cohnheim [1910] found that the residual volume of gastric contents 10 min. after a meal varied very little for iso- and hypotonic solutions.

In rats, Macleod and others [1930] found that, following the ingestion of hypotonic glucose solutions, the volume of the gastric contents rapidly diminished, although the glucose concentration remained almost

unaltered. This finding points to a rapid rate of pyloric outflow with little or no dilution of the gastric contents by secretion. Our experiments on dogs also show a rapid diminution in the volume of the gastric contents. It is probable that the failure of Carnot and Chassevant [1905] and Otto [1905] to demonstrate more rapid pyloric escape with hypotonic solutions may be associated with the use of duodenal fistulæ: in all experiments where the intact gastro-intestinal tract was employed, rapid emptying of hypotonic solutions was recorded. If absorption of water plays any part in the shrinkage in volume of the gastric contents, its effect should be more marked in the case of hypotonic solutions.

From the above considerations, it appears probable that the rapid resumption of the resting dimensions of the stomach following a hypotonic meal depends upon a rapid rate of pyloric outflow, little or no secretion of a diluting fluid and the tendency for an increase in the absorption of water by the gastric mucosa.

#### SUMMARY.

1. The emptying time of the stomach for hypertonic and hypotonic meals has been determined by the "outline method."
2. Hypertonic meals produce delay in proportion to the degree of hypertonicity.
3. Hypotonic meals leave the stomach more rapidly than isotonic meals.

One of us, W. R. Spurrell, is indebted to the Medical Research Council for a personal grant.

The expenses of this investigation have been defrayed in part by a grant from the Government Grant Committee of the Royal Society.

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612.122:547.472.3

## BLOOD LACTIC ACID IN MAN DURING REST.

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the London County Mental Hospitals.)*

THE problems connected with lactic acid production in the animal body have been extensively investigated by previous authors, but the obvious difficulties encountered in working with the intact organism have rendered the solution to some of these still a matter of speculation. When, for instance, the organism is subjected to a severe stress, the presence of lactic acid in the circulating blood can be readily understood, but its constant presence in small concentration, even during complete bodily rest, is less easy of interpretation. No satisfactory explanation has, in fact, yet been given either for the origin of blood lactic acid in the resting state or for the wide variations in its concentration. The existence of some muscular activity during bodily rest has led most authors to attribute the blood lactic acid to this source. In this paper, however, an attempt will be made to show that muscular activity can have little influence in this connection and that conversion of blood sugar (glycolysis) is the most probable source of this lactic acid. The wide variations in the resting levels of blood lactic acid will then be explained as one of the factors through which the organism endeavours to maintain a constant blood  $pH$ .

## EXPERIMENTAL.

In some of the experiments blood was taken from one of the superficial veins of the arm after initial compression, either manual or by tourniquet, which was released as soon as the needle was in the vein. The subjects were normal young adult males. In other experiments the femoral artery, femoral vein and jugular bulb were punctured, no compression being used. The subjects of these experiments are described in the text. The blood was collected into a small amount (approx. 0.03 g. per 15 c.c.

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blood) of Evans's 9:1 mixture of potassium oxalate and sodium fluoride, part of which was placed in the receiver and a smaller part on the barrel of the syringe. After thorough mixing the specimens were placed in a refrigerator or a portable ice-box.

Deproteinization was carried out by the Folin-Wu tungstate-sulphuric acid method. Actually 2 c.c. of whole blood were pipetted into a large centrifuge tube containing 14 c.c. of distilled water and 2 c.c. of  $2/3N$  sulphuric acid; 2 c.c. of sodium tungstate were then added and the contents well shaken before being centrifuged. The customary method is then to remove sugar by treatment with copper sulphate and lime. It was found, however, that this was an unnecessary procedure, since by the Friedemann method of lactic acid determination the amount of bisulphite binding material formed from the small amount of glucose present is negligible. On the other hand, when trichloroacetic acid was used as deproteinizing agent, it was usually necessary to treat the filtrate with copper sulphate and lime, not, presumably, to remove sugar, but to remove bisulphite-binding material other than lactic acid which remained in solution or suspension. The results recorded in Table I illustrate these points.

TABLE I.

Deproteinizing agent	Material	Lactic acid in mg./100 c.c. of whole blood	
		Filtrate treated with copper sulphate and lime	Filtrate used directly
Sodium tungstate and sulphuric acid	Sheep's blood	27.9	27.0
"	"	29.2	29.2
"	Human blood	12.6	12.6
"	"	14.4	14.9
"	"	17.7	17.7
"	Human blood after exercise	54.9	55.8
Trichloroacetic acid	Human blood	12.6	15.5
"	"	18.7	23.4
"	Human blood after exercise	54.4	59.1

Lactic acid was determined in 10 c.c. of the filtrate by the method of Friedemann, Cotonio and Shaffer, modified by Friedemann and Kendall [1929]. The apparatus used was that designed by Davenport and Cotonio [1927], except that the inlet tube of the boiling flask was connected by means of rubber tubing to the air outside the building. In these circumstances the "blank" value obtained was constant and amounted to 0.15 c.c. of 0.002*N* iodine solution. In all experiments at least two estimations were performed, the specimens being separated before deproteinization. In the great majority of cases, duplicate results agreed to within 0.2 c.c. of 0.002*N* iodine solution, *i.e.* approximately



2 mg./100 c.c. of lactic acid. Any experiment in which duplicate results showed a difference in excess of this figure was ignored. Similarly, in comparing the lactic acid levels of blood taken under varying conditions, differences below 2 mg./100 c.c. were regarded as insignificant. The percentage recovery of added lactic acid (as zinc lactate) was between 95 and 98.

On some of the blood samples *pH* measurements were made with the glass electrode apparatus designed by Kerridge, the precise technique employed being that previously described by one of us [Hurst, 1932]. For this purpose the blood was collected under paraffin.

*Blood lactic acid at rest.*

The introduction of the Clausen method in 1922 gave a fresh impetus to work on this subject, since previously no thoroughly reliable and convenient technique had been known. Using this method or one of its more recent modifications, numerous investigators have found that the resting levels of the lactic acid of whole blood of man vary within wide limits. Clausen [1922] gave results varying from 15 to 32 mg./100 c.c., whilst Owles [1930], with more refined technique, obtained results varying from 7 to 18 mg./100 c.c. The results of authors who used other methods for lactic acid determination are in close agreement and it seems clear that the normal resting levels differ by as much as 100 p.c.

In the present investigation 26 estimations on 9 normal subjects showed variations between 8.4 and 16.6 mg./100 c.c. (Table II). In each experiment the subject was in the post-absorptive state and was either in bed or had rested in an arm-chair for at least half an hour.

TABLE II.

Subject	Lactic acid in mg./100 c.c. blood			
	9.2	8.4	13.0	12.7
M. M. M.	9.2	8.4	13.0	12.7
I. H.	16.6	12.4	14.0	13.5
E. I.	14.3	11.7	13.2	9.0
A. M. T.	10.4	9.6	10.7	10.7
F. J. S.	13.3	10.2	12.1	—
P. C.	14.5	9.0	—	—
J. C. B.	10.1	12.6	—	—
F. P.	10.3	14.6	—	—
K. C. H.	9.2	—	—	—

The values are lower than those of previous authors except Owles; a fact which suggests that they were obtained by an improved technique and afford a truer index of the lactic acid content of blood. Table II shows also that even with the same subject under apparently identical conditions the variations are considerable. It seems probable that these wide variations are due neither to experimental error nor to changes in the blood during or after shedding.

POSSIBLE FACTORS INFLUENCING BLOOD LACTIC ACID  
PRODUCTION AT REST.(1) *Muscular contraction.*

It is known that lactic acid is constantly being produced in the muscles even during complete bodily repose. This was first shown in the case of frogs by Fletcher and Hopkins [1907] and amply confirmed by Meyerhof [1924] in the cases of amphibians and of mammals. It has usually been held that small amounts of the lactic acid so formed escape into the blood stream and that this supply constitutes the "resting" blood lactic acid. Against this hypothesis much evidence is accumulating. According to Hill, Long and Lupton [1924] the process of recovery after muscular exertion may be divided into two phases, the first being "nothing more than the oxidative removal of lactic acid in the muscles where it was formed before it has had time to escape into the blood and still further afield." This would appear to be the only phase necessary in very mild exercise, where lactic acid removal can keep pace with its formation. The second phase occurs when lactic acid formation is so rapid that some of it escapes into the blood stream, to be re-synthesized in the liver and in other muscles. Until recently the view that such moderate exercise as walking at rates of about  $3\frac{1}{2}$  m.p.h. produces a definite rise in blood lactic acid had not been questioned. This view depended mainly upon the work of Hill, Long and Lupton [1924], who found that a man walking at 3.5 m.p.h. for 28 min. raised his blood lactic acid from 20.9 mg. to 36.6 mg./100 c.c., whilst the same man walking at 4.1 m.p.h. for 33 min. raised it from 21.4 to 58.9 mg./100 c.c. Several other subjects were found by Long [1926] to give similar figures. Recent work by Owles [1930], however, has opened up the whole question as to the threshold at which lactic acid overflows into the blood after muscular exercise. Owles found in the case of two subjects in good training that walking for approximately 30 min. at speeds up to  $4\frac{1}{2}$  m.p.h. produced no rise in lactic acid in the blood taken from a superficial arm vein. At first sight these results suggested that, although no rise in lactic acid could be demonstrated in the blood from an antecubital vein, there might nevertheless be an escape of lactic acid from the leg muscles, but that the amount was insufficient to show a significant rise by the time it had been diluted in the general circulation and had passed through the inactive muscles of the arm.

In order to test the validity of Owles's results the following experiments were performed. A man of 48 (W. P. W.), who was accustomed to walking for about an hour every morning at a brisk pace (about  $4\frac{1}{2}$  m.p.h.), was made to walk at 4 m.p.h. for 30 min. Specimens of blood were taken from one of the antecubital veins at rest before exercise and from an antecubital vein and the femoral vein immediately after the exercise. The experiment was done on two separate occasions with identical results, which were corroborated in the case of another subject (B. T.), aged 55, who was accustomed to walking exercise and who walked for 30 min. at  $4\frac{1}{2}$  m.p.h. In neither case was there any rise in the lactic acid content of blood from the femoral vein, which demonstrates clearly that there was no escape of lactic acid into the veins immediately draining the muscles mainly involved in the exercise. Further experiments with

the subject B. T. showed that as soon as the rate of walking was accelerated to a speed incompatible with comfort, a rise in blood lactic acid occurred. Thus, after walking for 30 min. at  $5\frac{1}{4}$  m.p.h., it rose from 10 mg. to 23.5 mg./100 c.c. In the case of men not used to strenuous walking, an increase of blood lactic acid was obtained after walking at lower speeds. The figures obtained in the above experiments are shown in Table III.

TABLE III.

Subject	Age	Blood lactic acid in mg./100 c.c.			Rate and period of walking	Remarks
		Arm vein, resting	Arm vein, after exercise	Femoral vein, after exercise		
W. P. W.	48	16.9	17.8	16.9	30 min. at 4 m.p.h.	Good walker
		15.4	15.0	15.7	" " "	" "
B. T.	55	11.3	11.8	10.8	30 min. at $4\frac{1}{2}$ m.p.h.	" "
		10.0	22.0	23.5	30 min. at $5\frac{1}{4}$ m.p.h.	Considerable effort needed to maintain this pace
M. S.	62	11.0	19.5	20.5	20 min. at $4\frac{1}{2}$ m.p.h.	Fair walker for age
D. T. J.	24	10.5	20.5	21.0	24 min. at $4\frac{1}{2}$ m.p.h.	Very poor training

It appears from these experiments that the threshold of exercise beyond which an increase of lactic acid appears in the blood depends on the "fitness" or "training" of the individual, a condition which is determined partly by the constitution of the individual, but mainly by accustoming the organism to exercise. Training, according to Briggs [1920], develops the efficiency of the mechanism which supplies oxygen to the tissues. As Owles suggests, the power of the muscles to eliminate *in situ* the lactic acid formed during exercise probably depends upon the adequacy of the blood supply to them.

In the light of the above results it is unlikely that the muscles are unable to eliminate *in situ* the relatively small amounts of lactic acid they produce during bodily rest. It is even more unlikely that the amounts produced in the same person under apparently identical conditions of rest should vary so much as to cause the large fluctuations actually found.

Further evidence against the probability of the lactic acid content of the blood at rest being due to muscular activity was supplied by taking specimens from the femoral artery and the femoral vein at the same time. In no case was there any appreciable difference between the venous and

arterial sample (Table IV). This indicates clearly that the passage of blood through a large group of muscles during rest causes no significant change in its lactic acid content.

TABLE IV.  
Blood lactic acid in mg./100 c.c.

Subject No.	Blood lactic acid in mg./100 c.c.	
	Femoral artery	Femoral vein
1	12.6	12.6
2	11.5	13.4
3	16.3	14.7
4	12.6	14.4
5	11.9	11.9
6	9.7	9.0
7	13.5	11.7

The value of these observations, however, would be considerably diminished unless a definite rise in the lactic acid content of the femoral vein over that of the femoral artery could be shown in conditions where the leg muscles were known to be producing large amounts of lactic acid, for example, after vigorous exercise. At first sight this might appear to be an easy matter, but when it is remembered how rapidly the blood comes into equilibrium and how great an increase of the circulatory rate occurs after severe exercise, it becomes clear that at best no more than a small increase can be expected.

Recovery curves after severe exercise of short duration, such as standing-running "all out" for 1 min., show that the blood lactic acid rises rapidly for a few minutes after the exercise and then falls very much more slowly, regaining approximately its resting level in about an hour. The more severe the exercise, the longer is the period of ascent of the curve, the higher the maximum point and the longer the recovery period. The maximum, however, is always reached within 5 or 6 min. At this point the arterial and venous blood are in approximate equilibrium, and in order to show any excess lactic acid in the blood of the femoral vein, simultaneous arterial and venous punctures must be made immediately after the exercise. In order to demonstrate this, standing-running "all out" for 1 min. was carried out in three cases. The subject was first stripped and the femoral area cleaned up in order to save time. As soon as the exercise was completed the subject lay upon a couch, and the left femoral artery and right femoral vein were punctured simultaneously by two operators. Withdrawal of the blood was delayed until both operators were ready, and was then accomplished at the same rate and to the same amount. In two cases the blood had been transferred from the syringe to the receiving vessel within 3 min. In the third experiment a delay occurred owing to the femoral artery being entered instead

of the vein. This necessitated a change of syringe and the blood was ultimately withdrawn about 5 min. after the exercise. The results of these experiments, although too few to be convincing, were satisfactory (Table V).

TABLE V. Simultaneous punctures of femoral artery and femoral vein after severe exercise.

Subject No.	Blood lactic acid in mg./100 c.c.		Remarks
	Femoral artery	Femoral vein	
8	58.6	62.1	Within 3 min.*
9	71.8	74.8	Within 3 min.*
10	75.8	74.8	About 5 min.*

\* After completion of exercise.

In Cases 8 and 9 the blood was taken within the period of lactic acid increase and a slight but definite excess in the venous specimen appeared. In Case 10 the blood was withdrawn approximately at the lactic acid maximum and showed no significant difference between the arterial and venous figures. The subjects of these experiments (Nos. 1-10) were chronic mental defectives or psychotics in good physical condition.

It has now been shown that no increase in blood lactic acid is caused by the passage of blood through a large group of resting muscles nor by the taking of a moderate amount of exercise, as long as it is not severe enough to produce dyspnoea or discomfort. This evidence clearly indicates the error of assuming that muscular activity is responsible for the blood lactic acid during bodily rest.

It has also been shown that the blood draining a group of muscles, which have been vigorously exercised, contains slightly more lactic acid than that of their arterial supply, provided that the lactic acid maximum has not been reached. This indicates the possibility of demonstrating changes of lactic acid in the blood occurring during its passage through muscle, but only when the formation of lactic acid is very rapid and before its removal has begun to keep pace with its formation.

## (2) *Stimulation of the sympathetic nervous system.*

The effect of administration of adrenaline upon carbohydrate metabolism has been investigated in great detail, a review of the work being given by Cori [1931]. It has been demonstrated conclusively that injection of this substance leads to the breaking down of muscle glycogen without any associated muscular contraction, and that this results in the escape into the blood of large amounts of lactic acid.

The effects of subcutaneous injections on the blood lactic acid of man are shown in Table VI. It is known that excitement, fright or any emotion which powerfully stimulates the sympathetic nervous system

TABLE VI. Effect of 17 minims of 1/1000 solution of adrenaline hydrochloride given subcutaneously.

Subject	Blood lactic acid in mg./100 c.c.		pH		Pulse rate			
	Before adrenaline	20 min. after	Before	20 min. after	Before	10 min. after	20 min. after	30 min. after
F. J. S.	12.1	22.0	7.34	7.34	74	80	90	90
A. C.	13.5	24.8	—	—	No significant change			

increases the secretion of adrenaline with consequent rise of blood lactic acid. For this reason it might be suggested that the mood and emotional state of the individual, through their influence upon adrenaline secretion, are the regulators of the lactic acid content of the blood during rest. The present work, however, supplies little evidence in support of this view, since the blood lactic acid levels in the same subject appear to be entirely independent of mood. Furthermore, in experiments, not included in this paper, upon neurotic patients some of the highest levels were found in the most sluggish and phlegmatic of cases, whilst subjects showing sympatheticonia or suffering from anxiety states showed levels not appreciably above the average normal.

### (3) *Lactic acid production in the brain.*

The production of lactic acid in relatively large amount by the brain tissue has been shown to occur both *in vitro* [Warburg, Posener and Negelein, 1924] and *in vivo* [Holmes and Sherif, 1932]. The possibility of some of this lactic acid overflowing into the blood and thus constituting a part of the resting blood lactic acid had therefore to be considered. Specimens of blood were accordingly collected from the jugular bulb and from the femoral artery. It is obvious that the blood in the main arteries does not vary significantly, and for this reason the femoral artery, being the most convenient to enter without delay, was chosen in preference to the carotid artery. As not more than 2 min. elapsed between the removal of the venous and arterial blood, the samples may be taken as simultaneous for comparative purposes. The subjects were in the resting state and were of the same type as those used for other arterial punctures.

Table VII shows that the lactic acid level of the blood directly issuing from the brain is no higher than that of the arterial blood, that is to say, that all the lactic acid formed in the brain is dealt with *in situ* and plays no part in forming the resting blood lactic acid. The free passage of CO<sub>2</sub> into the blood, on the other hand, is demonstrated by the differences between arterial and venous pH values.

TABLE VII.

Subject No.	Lactic acid in mg./100 c.c. blood		pH	
	Jugular bulb	Femoral artery	Jugular bulb	Femoral artery
11	12.3	12.6	7.34	7.37
12	18.0	16.2	7.33	7.36
13	12.2	14.0	7.35	7.38
14	17.2	15.4	7.31	7.35
15	16.4	14.5	—	—
16	10.2	11.7	—	—

(4) *Threshold for lactic acid resynthesis.*

The possibility of a threshold below which lactic acid is not resynthesized by the muscles would afford an attractive hypothesis, in that it would solve in an easy manner the problem of the source of blood lactic acid at rest. The existence of such a threshold value would also account for the fact that the resting levels of the blood supplying and leaving a group of muscles on the one hand and the liver on the other are all approximately the same [Himwich, Koskoff and Nahum, 1930]. It would not, however, account for the wide variations obtained and there is at present no practical evidence to justify the assumption of such a threshold.

(5) *Glycolysis.*

The conversion of blood sugar to lactic acid *in vitro* has long been an established fact. The disappearance of glucose in shed blood was noted by Claude Bernard [1877], and has since been studied by many workers. Lovatt Evans [1922] has shown that large amounts of lactic acid are produced by glycolysis in shed blood and that the reaction is accelerated by alkali. It appears that the phenomenon is due to some extent to leucocytic activity and that it cannot occur in the plasma or serum alone. It is known to be accelerated by moderate increases of temperature or by raising the blood pH, to be retarded by decrease in pH and to be stopped completely by the addition of sodium fluoride in suitable concentration.

The existence of glycolytic activity in the blood *in vivo* is more difficult to prove, but considerable evidence is accumulating in its support. Eggleton and Evans [1930] have demonstrated in a lung-limb preparation of a dog a fall of blood sugar coincident with a rise of lactic acid, caused by raising the pH of the blood by means of sodium bicarbonate. Still more convincing is the work of Anrep and Cannan [1923] who found that in a heart-lung preparation an increase in pH led to an increase in blood lactic acid. In this case there could be little production of blood lactic acid by muscular activity, since it has been shown by Himwich, Koskoff and Nahum [1928] that the heart is more concerned with removing than with forming lactic acid. Anrep and Cannan considered the increase in lactic acid to be due more to interference with its oxidative removal than to its formation from sugar. Against this, however, it has been shown by Hartree and Hill [1924] that in the frog a fall in pH tends to retard lactic acid removal and that a rise in pH has no such effect.

In the present investigation, the effect of alkali ingestion upon man was determined, 20 g. of sodium bicarbonate dissolved in water being given by the mouth. The results recorded in Table VIII show that 30 min. after ingestion a small but definite increase in both blood pH

TABLE VIII. Ingestion of 20 g. sodium bicarbonate.

Subject	Blood lactic acid in mg./100 c.c.		pH	
	Before	30 min. after	Before	30 min. after
J. C.	13.0	15.8	7.31	7.35
W. S.	11.7	14.4	7.30	7.35
A. M.	11.5	13.7	7.31	7.36
M. H.	16.8	20.1	7.32	7.37

and blood lactic acid could be demonstrated. On the hypothesis that glycolysis is the source of the "resting" blood lactic acid, these results supply one at least of the reasons for the variations of blood lactic acid found in the same resting individual at different times. It is known that slight changes in  $pH$  are constantly taking place in the circulating blood, and it is to be expected from the above evidence that these are accompanied by changes not only in  $CO_2$  tension but also in lactic acid concentration. Thus glycolytic activity, stimulated by a tendency to increased blood  $pH$  and depressed by a tendency to decreased blood  $pH$ , offers a material basis for the suggestion of Anrep and Cannan [1923] that blood lactic acid changes serve as a third line of buffer resistance, and for the statement of Peters and van Slyke [1931] that (in states of alkalosis) the "lactic acid increase must be looked upon as an adaptive reaction, as it tends to mitigate the alkalosis to which it is a response."

#### SUMMARY.

1. The technique of lactic acid estimation by means of one of the more recent modifications of Clausen's method is described. It is shown that the removal of sugar is unnecessary as long as tungstate-sulphuric acid deproteinization is used.

2. An attempt is made to trace the source of blood lactic acid in man during bodily rest. Resting blood lactic acid values observed by previous authors are reviewed and are compared with the figures obtained in the present studies. The results show that any satisfactory explanation of the existence of this lactic acid must also account for the wide variations observed in the same individual at different times, but under apparently identical conditions of bodily rest.

3. Possible sources of blood lactic acid at rest are discussed and experimental evidence is presented in support of the following conclusions:

- (a) During bodily rest the muscles supply no lactic acid to the blood. In the case of men in good training, walking at speeds up to  $4\frac{1}{2}$  m.p.h.



for 30 min. produces no increase in the lactic acid concentration of blood drawn from the femoral vein.

(b) Activity of the sympathetic nervous system with consequent secretion of adrenaline is an unlikely source.

(c) Comparison of blood samples taken in immediate succession from the jugular bulb and from an artery during rest shows that the blood receives no demonstrable amount of lactic acid from the brain.

(d) The existence of a threshold value below which lactic acid is not resynthesized by the muscles is an hypothesis which lacks supporting evidence of a practical nature and which would still leave unexplained the wide variations in lactic acid concentration.

(e) Conversion of blood sugar (glycolysis) is the most probable source of the blood lactic acid at rest. The evidence of glycolytic activity, shown by previous workers to take place *in vitro* and in heart-lung preparations, is supported by experiments *in vivo*, in which an increase in blood pH by means of alkali ingestion is shown to increase the blood lactic acid concentration. These experiments also suggest that the variations in lactic acid concentration during bodily rest are due to stimulation or depression of glycolytic activity and assist the organism in its endeavour to maintain a constant blood reaction.

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## ADRENALINE IN THE SUPRARENAL MEDULLA.

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OF the many chemical methods which have been described for the estimation of adrenaline in extracts from the suprarenal glands, most depend on the use of oxidizing reagents to produce a red-coloured product of the partial oxidation of adrenaline itself. The method of Folin, Cannon and Denis [1912], on the other hand, depends on the blue colour produced in an alkaline phosphotungstate mixture by the reducing action of adrenaline. There is an obvious possibility that derivatives of adrenaline, not possessing its physiological activity, might, if present in an extract, contribute to the red colour produced by oxidizing reagents; while reducing substances other than adrenaline will produce a blue colour with Folin's reagent. It is not surprising, therefore, that several investigators have drawn attention to cases in which the colorimetric estimate of adrenaline in an extract differed significantly from that obtained by a physiological determination of activity; though, in general, the results obtained with extracts from fresh, normal glands have usually shown good correspondence, as in the original comparisons made by Folin, Cannon and Denis [1912]. Where discrepancies have occurred, these have usually been in the direction of over-estimates by the colorimetric test. Kojima, Nemoto, Sato, Saito and Suzuki [1932], on the other hand, recently drew attention to another kind of discrepancy, apparently due to the presence of other physiologically active substances in an extract, causing differences also between the physiological estimates obtained by different methods.

Such discrepancies as have hitherto been recorded are of different kinds, and of much smaller dimensions than that recently described by Annau, Huszak, Svirbely and Szent-Györgyi [1932]. These authors examined extracts from the separated medulla of ox suprarenal glands, rapidly frozen as soon as possible after slaughter, and kept in a frozen condition until the extract was prepared by a method designed to avoid decomposition of the natural hormone. In such extracts they observed a

physiological activity corresponding to that of about ten times the quantity of adrenaline estimated from the colour produced by the oxidizing action of iodine. The existence of the discrepancy was confirmed by other colorimetric tests. It could be interpreted as due either to the presence of some substance or condition hindering the oxidative production of the coloured derivative, or to the existence of adrenaline in the suprarenal medulla as an unstable derivative having many (at least 10) times the activity of the free substance, as isolated by chemical methods. Szent-Györgyi and his co-workers chose the latter interpretation, and provisionally gave the name "novadrenine" to this active derivative, in which they supposed adrenaline to occur naturally, as the true, unchanged suprarenal medullary hormone. Their choice appears to have been determined by two considerations. The physiological action of their medullary extracts, if due to free adrenaline, would have indicated the presence of as much as 15 to 30 mg. of adrenaline in 1 g. of fresh medullary substance. Apparently they regarded such a high content as unthinkable. Further, in attempting to isolate the supposed novadrenine, they found that the values determined by colour and physiological tests would, at some stage of purification, become newly concordant. They attributed this to liberation of adrenaline from the unstable novadrenine; but, in the absence of any quantitative data, to show that the new match was reached by loss of physiological action rather than by gain of colorimetric value, the fact recorded could as easily be explained by removal of hindrance to oxidative colour production.

The experiments here recorded were undertaken to obtain further evidence as to the meaning of the discrepancy described by Szent-Györgyi and his co-workers<sup>1</sup>.

#### METHODS.

*Material and extraction.* Szent-Györgyi and his co-workers laid great stress on the protection of the suprarenal glands from *post-mortem* change by cold, and on the use of what they regarded as a conservative method of extraction. Our precautions were probably at least as strict. They state that the glands (ox) were excised for them as soon as possible after death, and transported on ice to the laboratory, where they were frozen before dissection and mincing. We arranged to have ox glands from

<sup>1</sup> Since this paper was accepted U. S. v. Euler has published in this *Journal* (78, 462) an account of a shorter investigation, by which he also was led to conclude that the discrepancy in question had no true significance, being probably due to some defect in the colorimetric method used by Szent-Györgyi and his colleagues; and this conclusion has been accepted by Professor Szent-Györgyi, in a footnote to v. Euler's paper.

the abattoir removed as soon as possible, and frozen with solid  $\text{CO}_2$  before transport to the laboratory. In other cases we were able to use glands from horses slaughtered at the Institute's farm; and these could be obtained within a few minutes of slaughter, and then similarly frozen for transport. We found no evidence, indeed, that this accelerated excision enabled us to obtain extracts of higher activity than those yielded by glands removed at the abattoir after a longer interval. In any case, however, our failure to confirm the suggested discrepancy between colorimetric and physiological values was not due to the neglect of precaution against *post-mortem* change. As a rule the hard frozen glands were dissected as soon as received in the laboratory; but in some cases they were kept for periods up to 2 weeks at about  $-4^\circ \text{C}$ . before use. Glands so stored yielded as active extracts as those obtained from others of the same batch worked up immediately on delivery.

The frozen glands were cut open, and the medulla dissected as cleanly as possible from the cortex without allowing it to thaw. We did not use a mincer, but transferred the medullary substance to a mortar containing the cold extraction fluid and clean sand, with which the substance was quickly ground to a fine suspension. Three methods of extraction were used.

(i) Szent-Györgyi's method, of extracting with cold 0.5 p.c. trichloroacetic acid, raising the mixture briefly to  $70^\circ \text{C}$ . to complete the coagulation, and then cooling the filtered extract and preserving below  $0^\circ$ .

(ii) An alternative method, mentioned by Szent-Györgyi as equivalent, of extracting with 5 p.c. trichloroacetic acid in the cold. At this strength the cold acid itself is an efficient protein coagulant, and no heating is required. After filtration the excess of trichloroacetic acid can be removed, as suggested to us by Dr Dudley, by shaking with several successive quantities of ether, carefully purified from peroxides. The ether also removes traces of lipid substances, which make the extract slightly opalescent and hinder colour comparisons. Shaking with ether is repeated until the extract is perfectly clear and colourless, and is only just acid to congo-red paper. The extract is then preserved below  $0^\circ \text{C}$ .

(iii) The familiar method of extracting with 0.5 p.c. acetic acid, boiling briefly to coagulate proteins, and filtering.

It will be seen later that the results obtained by the three methods of extraction are practically identical; so that our experience affords no ground for supposing that the method of extraction used by the Hungarian workers had any special effect in preserving a natural adrenaline complex.

*Methods of colorimetric determination.* With the exception of the Folin method, in which adrenaline acts as a reducing agent to produce a blue-coloured derivative of phosphotungstic acid, the different methods which have been described for the colorimetric determination of adrenaline depend on the formation of a red product of partial oxidation. Szent-Györgyi and his co-workers used a method of this kind, depending on the production of the red colour by oxidation with iodine, as originally described by Vulpian. They treated the extract to be tested and a control solution of pure adrenaline simultaneously with Lugol's solution, added in sufficient excess to colour the solution with free iodine; the free iodine was then removed by sodium thiosulphate and the colorimetric determination immediately made by direct comparison of the two solutions. The value obtained was controlled by other tests, including that based on oxidation by potassium persulphate, introduced by Ewins, and identical results are stated to have been obtained. In all cases the determination was based on direct comparison of the colour produced in the extract with that in a control solution of pure adrenaline.

In our own experiments the persulphate test was first tried, on account of the facility which it offered, by the use of a colourless reagent, for studying the course of the development of the colour in the two solutions. We were immediately struck by the extraordinary slowness with which the colour appeared in the gland extract. In the pure adrenaline solution the colour appeared rapidly, and the oxidation had passed far beyond the stage of maximum red colour at a time when the red colour was in the initial stage of its slow development in the similarly treated gland extract. No kind of estimate of the adrenaline in the extract could be obtained by direct comparison under such conditions. Means could be found, indeed, as described below, to reduce this disparity in time relations, but without any certainty of its elimination. There was a further hindrance to the accuracy of direct comparison, in the fact that the colour produced by partial oxidation of adrenaline, by any reagent, is a mixed colour, with a red, a yellow and a blue component, which may to some extent vary independently; so that, even if the process could be so modified as to produce certainly simultaneous colour maxima in the pure adrenaline solution and the gland extract, the direct comparison would still be liable to difficulty and inaccuracy on account of minor qualitative differences between the colours.

For these reasons we abandoned the attempt to make estimates by direct comparison in a colorimeter of the Dubosq type, with any of the colorimetric methods. Using the Lovibond-Rosenheim [1927]

tintometer, in which the colour of the solution is rapidly matched by an appropriate combination of standard red, yellow and blue glasses, we could determine, for adrenaline solution and for extract alike, the strength of the red component of the colour at its maximum. An accurate and reproducible comparison could thus be made, even when the maxima were not simultaneous. It was only necessary to choose conditions which would lead to the development of the colours in the two solutions at rates not too widely different, and of the different colour components in approximately the same ratios.

The three tests thus used by us were the persulphate test of Ewins, as modified by Barker, Eastland and Evers, the iodine test chiefly used by the Hungarian observers, and the Folin test.

*The persulphate method.* In a recent examination of the development of the colour in this test, Barker, Eastland and Evers [1932] showed that the rapidity of the development and the time of persistence of red colour were favoured by the presence of the chloride ion, by a *pH* between 5 and 6, and by the presence of a trace of a heavy metal, in particular of copper. Having confirmed these authors on these various points by our own experience, we have used, in all our own determinations by this method, a buffered persulphate reagent, made up in accordance with their indications, to the following formula, with a *pH* 5.5:

NaCl	...	...	...	...	1
K <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	...	...	...	...	0.2
Na <sub>2</sub> HPO <sub>4</sub>	...	...	...	...	0.239
NaH <sub>2</sub> PO <sub>4</sub>	...	...	...	...	0.937
Water to 100.					

To the extract or control adrenaline solution the persulphate reagent was added in equal volume. The mixture, at the temperature of the laboratory (18°–20° C.), was placed in the tintometer and successive matches made with the coloured glasses until the maximum value for the red component was reached.

The sensitiveness of the reaction to traces of heavy metals makes it difficult to control the rate at which the colour develops. The use of a syringe with a steel needle, instead of a glass pipette, to measure a small volume of an extract, was found, on one occasion, to produce a great acceleration of the colour development. We tried the effect of deliberately accelerating the development by dipping a strip of clean copper into the mixture of extract and persulphate reagent, and found that the colour, as expected, reached its maximum much more rapidly, but that the

maximum so attained was somewhat lower than that ultimately reached in the same mixture without copper.

Apart from such known and deliberate changes of conditions, affecting the rate of colour development, we observed changes in the rate which appeared to be capricious, and were due to causes which we were unable to identify. We observed, on one occasion, that a freshly prepared medullary extract showed a very pronounced retardation of the development of maximum colour with the persulphate reagent, and that the inhibiting factor had, apparently, largely disappeared when the extract was tested again after being kept for a day or two, the same maximum being then attained, but with a rapidity comparable to that seen with pure solutions of adrenaline. Later, however, the same extract again showed a retarded colour development. In successive tests made on one extract, even at different times during the same day, widely varying periods were required for the attainment of the colour maximum. Attempts to associate these variations with changes in illumination or other conditions were unsuccessful. The reaction is extremely sensitive to traces of heavy metals, as already indicated; and this probably explains the fact, observed by us, that the colour development is greatly retarded by adding potassium cyanide to the persulphate reagent, in dilutions as low as one-fiftieth of the molar concentration of the persulphate. Even pure solutions of adrenaline are to some extent subject to apparently capricious variations in their rate of oxidation by this reagent; but these are much more prominent in dealing with gland extracts.

Whatever may be the sources of these irregularities, they appear to us to be of such a kind that there would be little hope of eliminating them, under the working conditions of an ordinary laboratory. When the time of maximum development of the colour in an extract may vary from 5 min. to an hour, and when the average time required by extracts to reach the maximum is about three times that required by a pure solution of adrenaline, it is clear that direct comparison of the colour developed in an extract with that of the control solution at its maximum can only give grossly misleading results. Under such conditions we should have found it easy to obtain measurements comparable to those of Szent-Györgyi and his colleagues, suggesting that an extract contains adrenaline in only about one-tenth of the quantity corresponding to its physiological action. On the other hand, although the proportion of yellow to red in the colour at its maximum tends to increase somewhat with the time of development, the readings of maximum value of the red component by the Lovibond-Rosenheim tintometer showed but slight variation



with the same extract, even over a fairly wide range of times taken in reaching the maximum. By comparison of these maximum red values with those of a pure adrenaline solution, satisfactorily consistent estimates of the adrenaline content of an extract could be obtained by the persulphate method. And these, again, agreed fairly well with the estimates obtained by the iodine method, when both were used for the same extract.

*The iodine method.* This was the method which gave the results on which the conclusions of Szent-Györgyi and his co-workers were mainly based. The course of the development of the colour in this reaction can be studied by adding iodine (Lugol's solution) in distinct excess to a series of samples of an adrenaline solution, and removing the excess of iodine with thiosulphate at different intervals after its addition. In the earliest stage the colour so revealed is a yellowish red, which, in samples from which the iodine is discharged later, becomes successively a pure red, and then a bluish red. The rate at which this sequence of changes occurs is conditioned by the hydrogen-ion concentration. At pH 6.2 the following sequence can be observed by the tintometer. If the excess of iodine is removed as soon as possible after addition, a colour is observed containing about 85 p.c. red to 15 p.c. yellow by the Lovibond glasses. If the excess of iodine is allowed to remain for 50–100 sec. before discharge, the colour obtained is a "pure" red—i.e. one requiring no admixture of yellow for matching in the tintometer—and the intensity of this is the same, within about 5 p.c., as that of the red component in the earlier mixture. If the iodine is allowed to act still longer, a blue component replaces the yellow in the resulting colour, while the red component still remains approximately constant. Finally, if the excess of iodine is allowed to act for 15 min. or more, both blue and red components diminish. In solutions more strongly acid than pH 6, the yellow component is stronger in the early state, and the development to the blue-red stage requires a longer action of the iodine. If the acidity is still greater (pH less than 4) the red colour develops very slowly. On the other hand, as the reaction is shifted to the alkaline side of pH 6, the sequence of changes is accelerated, becoming so rapid at a definitely alkaline reaction that it is difficult to be certain of stopping the reaction in the maximum range; and the colour, after discharge of excess of iodine, fades so rapidly in an alkaline mixture that it is not easy to obtain a stable and satisfactory reading<sup>1</sup>.

A large excess of iodine in a strongly acid solution appears to destroy the red component faster than it is formed, so that a red solution is never

<sup>1</sup> Cf. v. Euler, *loc. cit.* p. 465.

obtained. At pH 6, on the other hand, a moderate excess of iodine makes no difference to the strength of the red colour obtained in the same time of action. At reactions more acid than about pH 6 the production of the colour maximum by iodine was always slower, though to varying degrees with different extracts, than with pure solutions of adrenaline. When, on the other hand, the reactions of both were in the neighbourhood of pH 6, there was no perceptible difference between the rates of colour production in a pure solution of adrenaline and in medullary extracts. In contrast with the persulphate test, and also with that of Folin, the rate of colour development in the iodine test appears to be unaffected by traces of heavy metals.

For these various reasons it was clear that a reaction in the neighbourhood of pH 6 provided the optimum conditions of colorimetric determinations by this method. Szent-Györgyi and his co-workers state that their extracts were "neutralized with a small excess of sodium bicarbonate." This would appear to indicate the choice of a reaction far too alkaline, in our experience, to enable stable and consistent readings to be obtained by the iodine method; but we are unable to judge to what extent this accounts for the difference between their results and our own. Our own procedure was as follows:

The control solution was made by dissolving an accurately weighed quantity of pure adrenaline base with the aid of a trace of dilute HCl, and diluting with distilled water. The reaction of the medullary extract to be tested was pre-adjusted, if necessary, to the neighbourhood of pH 6. A preliminary determination indicated the approximate strength of adrenaline to be determined. Appropriate dilutions of the control solution and of the extract were then made with a 1/15 molar phosphate buffer solution, made up according to Clark's directions, with a pH 6.2. Lugol's solution, diluted to a strength of  $N/100$  iodine, was then added, in the proportion of 1 c.c. of the dilution to about 0.1 mg. of adrenaline. The mixtures were allowed to stand for equal times, in the range from 50 to 100 sec., experience having shown that within this range a stable colour was produced, which could be matched by the red glasses of the tintometer without introduction of a yellow or blue component. The determination can be made rapidly, and successive readings on the same solution or extract do not vary by more than  $\pm 3$  p.c. Since the colour develops at the same rate, under these conditions, in pure adrenaline solutions and gland extracts, and has the same pure quality in both cases, direct comparisons can also be made with a colorimeter of the Dubosq type.

*Folin's test.* In contrast to the other tests, in which adrenaline acts as the specific parent substance of the coloured product on which the measurement is based, Folin's test depends on the reducing action of adrenaline, in producing a coloured product of phosphotungstic acid. This reducing action is not specific. It is produced by physiologically inactive oxidation products of adrenaline, by uric acid, and, according

to Szent-Györgyi, by hexuronic (ascorbic) acid, which is a constituent of the suprarenal cortex.

There seem, therefore, to be many chances of the Folin test, when applied to suprarenal extracts, giving an estimate in excess of the true adrenaline content; and several investigators have stated, indeed, that extracts of the whole gland do give estimates higher than the true value [Lewis, 1916; Frowein, 1922; Maiweg, 1922; Watanabe and Sato, 1928]. On the other hand, a careful comparison made by Folin, Cannon and Denis [1912] at the time of the first description of the test, showed a very satisfactory correspondence between the adrenaline contents determined by it for a series of gland extracts, and the physiological estimates made on the same extracts.

Baker and Marrian [1927] have shown that the development of the colour in the Folin test takes a time which varies according to conditions, the presence of traces of heavy metals having, in particular, an accelerating action. In order to see the course of the development it is better to dilute the mixture of phosphotungstate and adrenaline-containing solution to near the final volume, before adding the sodium carbonate and thus starting the reducing action. It is then seen that the blue colour reaches about 85 p.c. of its maximum in about 3 min. The maximum is then more slowly approached, being reached only after about 10 min. Later, the colour slowly fades again. The colour is mainly blue, but the presence of a yellow component increases the difficulty of matching it in the Lovibond tintometer. On the other hand, the development of the colour is not delayed in the gland extract by the presence of non-specific constituents, so that direct comparison with the control solution, as in the ordinary performance of the test, gives good results.

Most of the criticisms of the test, as giving unduly high values through the presence of non-specific reducing agents, have been based on experiments with extracts from the whole suprarenal gland, including the cortex. We were dealing with extracts from the separated medulla, and these were so prepared as to be free from products of the partial oxidation of adrenaline. On the other hand, it is impossible to be certain of removing every trace of cortical material in dissecting out the medulla from the frozen glands. It might, accordingly, have been expected that our estimates on these extracts by the Folin test would give rather high values. As a matter of fact, it will be seen that, when the Folin test was applied, the estimate of adrenaline which it gave was consistently lower than that obtained on the same extract by the iodine method, and

usually lower than that given by the persulphate method. We are unable to explain the discrepancy, and can only record it.

*Physiological determinations.* For these we used only the test on the arterial blood-pressure reaction of the spinal cat. The cord was cut under ether at the level of the second cervical vertebra, and the cord anterior to this section, the bulb, and the whole of the brain completely destroyed. The determinations of the adrenaline content of an extract were made in the usual way by finding a dose producing a rise of arterial pressure matching, as exactly as possible, that produced by a submaximal dose of a control solution of pure adrenaline. The most favourable conditions for accuracy were presented by a preparation in which, some hours after cord section, the "resting" arterial pressure had attained a low, steady level. By bracketing doses the adrenaline activity of an extract could usually then be estimated to  $\pm 5$  p.c.

The general nature of the results soon became clear. When injections were, in the ordinary way, made into a peripheral vein, the adrenaline determined in an extract, in comparison with a pure adrenaline solution, was definitely in excess of that determined by any of the colorimetric methods used by us. The difference, indeed, was not of the order suggested by Szent-Györgyi and his co-workers. They found an activity at least 10 times as great as that indicated by the colour test. We found an excess activity corresponding to only 10–40 p.c. of the adrenaline determined by colorimetry; but it was generally present. An inspection of the arterial pressure records showed, however, a qualitative as well as a quantitative difference between the changes produced by equicolorimetric doses of pure adrenaline and of the gland extract respectively. The curve shows, in either case, a very rapid rise to an obvious "shoulder," from which the pressure climbs more slowly to the brief maximal "pinnacle." It could frequently be seen that the excess of the maximum rise produced by the dose of extract, over that produced by the colorimetrically equivalent dose of pure adrenaline, was entirely due to the second phase of the rise—a higher "pinnacle" being reached from an equally high, or even a lower "shoulder." Since this second phase involves, and is partly due to acceleration and augmentation of the heart beat, it seemed possible that some other constituent of the complex extract, by a reinforcing or steadying action on the heart beat, might assist in raising the blood-pressure to a level above that to which it would be driven by the unmixed action of the adrenaline present in the dose. This possibility could be tested, by injecting the doses of adrenaline and of gland extract into the thoracic aorta, so that they would pass

directly to the peripheral arterioles and produce their main pressor effects by vaso-constriction, effects on the heart being reduced to those of such traces of adrenaline or other substance as might pass through the peripheral vessels, and delayed by a whole circulation time. For this purpose it was necessary to use a long capillary glass cannula, introduced through the cut central end of the right carotid artery, and pushed down so that its tip rested against the inside of the lesser curvature of the aortic arch, as described by Dale and Richards [1927]. The blood had to be made incoagulable by heparine, and this involved a modification of the method of destroying the brain. By a preliminary dissection under ether both carotid arteries were tied, and then, the upper part of the sternum being split without opening the pleuræ, the vertebral arteries were tied in succession, and the subclavian arteries central to the origin of the vertebrals. The tying of the second vertebral artery was followed by a few deep gasps precluding the complete cessation of breathing. Artificial respiration being applied, the cord could now be cut, and the brain destroyed, as a completely bloodless operation. After all further dissection had been completed, and arrangements made for inserting the capillary aortic cannula, heparine could then be injected without any fear of hæmorrhage from the intracranial vessels.

## RESULTS.

The results are assembled in Table I, which enables the estimates obtained by the different colorimetric and physiological methods to be compared.

TABLE I. Mg. of adrenaline per g. of fresh, moist medulla.

Date	Animal	Colorimetric			Physiological	
		Iodine	Per-sulphate	Folin	Intra-venous	Arterial
Dec. 23rd, 1932	Horse	(a) 8.8	—	—	9.87 9.49	—
Jan. 2nd, 1933	Ox	(a) 5.68	5.4	5.12	7.33 7.74 7.33 7.94	5.12
Jan. 16th, 1933	Ox	(a) 8.43 (b) 8.18 (c) 7.33	—	—	12.0	6.74
Jan. 27th, 1933	Ox	(a) 9.86	—	8.88	12.28	7.9
Feb. 2nd, 1933	Ox	(a) 6.85 (b) 8.09	—	—	—	—
Feb. 15th, 1933	Horse	(a) 10.74 (b) 11.47	10.74 10.50	9.81 9.93	10.74 12.62	—

The following points may be noted:

(1) When colorimetric determinations are made by more than one method on the same extract, the results are in fairly good agreement, but the estimates by the Folin method are, on the whole, lower than those by the iodine method.

(2) The samples used on January 2nd and 16th were prepared from a batch of material collected on the former date. It will be seen that the extract made on the day of collection was much weaker, by all tests, than that made from other glands of the same batch, kept for a fortnight before extraction. Probably the glands chosen for immediate extraction happened to have smaller stores of adrenaline; in any case there seems no reason to doubt the stability of glands kept below 0° C.

(3) The three different extraction methods above described were used in certain cases. In Table I (*a*) indicates an extract made with cold 5 p.c. trichloroacetic acid, the excess being removed from the filtrate by ether; (*b*) an extract made, by Szent-Györgyi's usual method, with 0.5 p.c. trichloroacetic acid and brief heating to 70°; (*c*) an extract made by brief boiling with 0.5 p.c. acetic acid. It will be seen that there is no regular excess of adrenaline shown by any one process over the other when used on samples from the same batch of glands. Whenever the comparison was made, the extract by simple boiling with dilute acetic acid showed, further, a similar excess of activity by intravenous injection over the colorimetric determination to that shown by the extracts made from the same batch by either of the trichloroacetic acid methods recommended by the Hungarian workers. There seems no ground, in any case, for the suggestion that activity, as distinguished from adrenaline content, is lost by the simple, ordinary method of extraction.

(4) The adrenaline contents determined by the intravenous physiological test range from 7.33 to 12.62 mg. per g. of fresh medulla. None of these values reaches the range of 15–30 mg. per g. mentioned by Szent-Györgyi and his co-workers. On the other hand, the values are not of a lower order. According to their statement, their colorimetric values were not more than one-tenth of the physiological. Their colorimetric estimates, accordingly, showed not more than 1.5–3 mg. per g.; and these values are more definitely inferior to those obtained in our colorimetric determinations (5.7–11.5) than are our physiological determinations to theirs.

(5) Our physiological estimates, obtained by matching the heights of blood-pressure rises with intravenous injections, almost always showed a definite excess over the colorimetric estimates on the same extracts, as

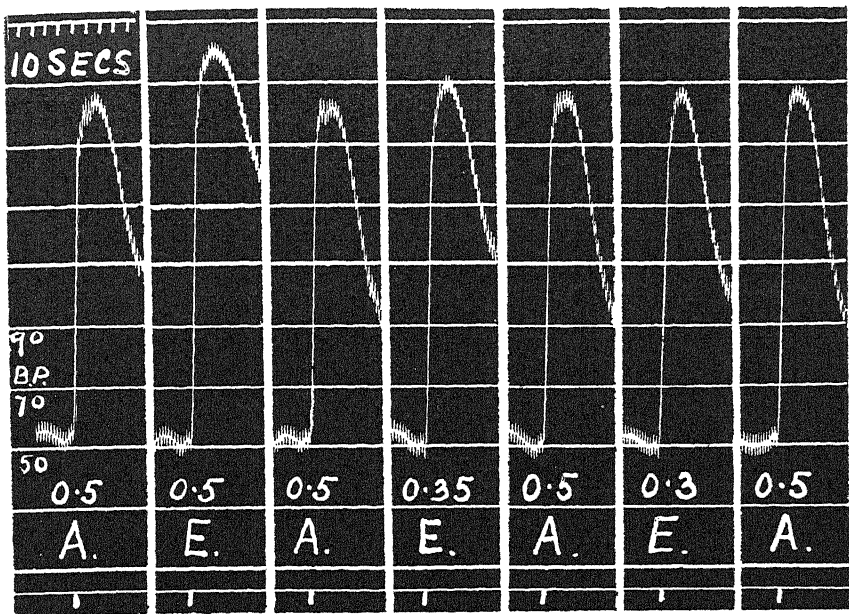


Fig. 1a. Experiment of January 11th. Comparison of diluted extract (*E*) with equi-colorimetric solution of adrenaline 1 : 50,000 (*A*), on spinal cat with venous injections.  $0.35 E > 0.5 A$ .  $0.3 E = (\text{approx.}) 0.5 A$ .

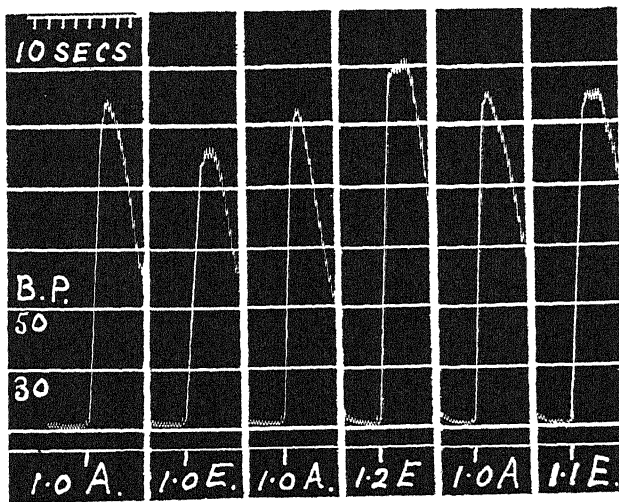


Fig. 1b. Continuation of experiment, with arterial injections into the same cat.

$$\begin{aligned}
 &1.0 A > 1.0 E \\
 &< 1.2 E \\
 &= 1.1 E. \therefore 0.55 E = (\text{approx.}) 0.5 A.
 \end{aligned}$$

has been mentioned already. The experiment of February 15th shows that the discrepancy is not always present. The discrepancy is, in any case, of a relatively small order, and has no relation to a difference of the kind described and illustrated by Szent-Györgyi and his co-workers. The estimates made by us on January 2nd, 16th and 27th with intra-arterial injections show a reversal of the discrepancy, the physiological estimates by this method being, in all cases but one, as definitely inferior to those made by colorimetry, as are the latter to the physiological estimates with ordinary intravenous injection. Fig. 1 is taken from part of the record representing the comparisons made on January 2nd.

#### DISCUSSION.

The discrepancy which we have observed has no connection with that which we set out to investigate. The fact that it appears in opposite directions, according as the physiological tests are made with one or another form of injection, may be taken to indicate a limit to the accuracy with which the actions of a crude extract and of pure adrenaline can be compared. The comparison is clearly subject to interference by some substance which potentiates the pressor effect of adrenaline with intravenous, and antagonizes it with intra-arterial injections. The simplest conception is of a substance, or mixture of substances, which—at least when adrenaline is also present—improves the output of the heart by a direct action on that organ, and has also a peripheral vaso-dilator action, antagonistic to the vaso-constrictor effect of adrenaline. A comparatively very weak activity of this kind would suffice to modify the adrenaline effect in opposite directions with the two methods of injection, so as to produce the disparity between the two estimates, and between either and the colorimetric estimate, which presumably gives the nearest approximation to the true adrenaline content, at a value somewhere between the two physiological estimates.

We find no evidence at all for the existence of a real, major discrepancy of the kind described by Szent-Györgyi and his colleagues. It seems probable that the low colorimetric values which they obtained were due to various conditions which cause the coloured product of partial oxidation to appear more slowly in the impure extract than in the pure adrenaline solution, or which otherwise produce a false reading of the adrenaline content when direct comparison is made between the simultaneous colorations of the two solutions. Our results, it will be seen, eliminate the large discrepancy, not by depressing the physiological estimates to meet colorimetric values as low as theirs, but by raising the



colorimetric values, determined under fairer conditions by reading each at the maximum, to meet physiological estimates of the same order as theirs. The highest value we obtained, by physiological assay, was, indeed, not much above 12 mg. of adrenaline per g. of fresh medulla. We find no reason to doubt, however, that, with certain samples of glands, we should obtain extracts of a physiological activity corresponding to 15 mg. or more of adrenaline per g., or that colorimetric tests, properly applied, would show that activity to be due to adrenaline itself. Adrenaline contents of this order in the separated medullary substance are not, as Szent-Györgyi and his co-workers have assumed, "impossible." The tenfold discrepancy observed by them was clearly due, in our opinion, to their underestimating, to that extent, the amount of adrenaline actually present, and not, as they supposed, to its presence as novadrenine, with an activity at least ten times as great.

#### SUMMARY.

The existence of adrenaline in the suprarenal gland as a complex, novadrenine, of enhanced activity, is not confirmed. Adrenaline exists in the gland in a quantity corresponding to the physiological estimate.

I wish to express my sincere gratitude to Sir Henry Dale for his continual help and advice during this research.

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## THE INFLUENCE OF CERTAIN FACTORS UPON THE VOLUME OF THE INTRATHORACIC VENÆ CAVÆ.

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It is probable that many factors affect the volume of the intrathoracic venæ cavæ of mammals during respiration; in this paper only two will be considered in detail, namely, structure and intramediastinal pressure. The thoracic inferior vena cava of the dog, cat, and rabbit [Franklin, 1931, 1932, 1933] lengthens during inspiration, but the superior vena cava does not generally do so in quiet breathing, although its structure in the cat and in the rabbit would permit such lengthening if it became necessary. If the thoracic inferior vena cava reaches its limit of lengthening, the heart is pulled caudally and the trachea and superior vena cava lengthen a little; inspiration, however, is becoming rather forcible when this stage is reached. The structure of the thoracic inferior vena cava in the dog, cat, and rabbit imposes variations in volume according to its length, and when other factors, such as respiratory variations in intramediastinal pressure, are excluded, the volume is found to be greatest at the expiratory length of the vessel. If, however, as is generally accepted, the intramediastinal pressure decreases during inspiration, this will tend to increase the volume of the vein and so will oppose the effect produced by its structural peculiarities. The primary object of the present research has been to investigate the combined effect of these two opposite influences upon the vein volume. At the same time, the volume changes of the dog's superior vena cava, when exposed to varying pressures, have been recorded. The structure of the latter vein in this species gives no anatomical justification for supposing that it undergoes functional changes in length; it differs, therefore, in this respect from the corresponding vessel in the cat and in the rabbit.

Many difficulties have arisen during the work, and of these two in particular must be mentioned here. The first is that it is not easy to obtain from the literature any satisfactory quantitative conception of the intramediastinal pressure changes in different species of mammals. It is very

doubtful if "intrapleural" pressure records can give any indication of intramediastinal values [Rehfish, 1927], and even van der Brugh's technique [1900] leaves one somewhat sceptical. Meltzer [1892] essayed the measurement of intramediastinal pressures in large rabbits, and he found that the pressure changes were extremely small except at the caudal end of the mediastinum, so that only the diaphragmatic end of the thoracic inferior vena cava was exposed to pressure variations of any magnitude. From a study of all the available data, one gathers the impression that the variations in pressure within the dog's mediastinum during ordinary breathing are very unlikely to be as great as 20 cm. H<sub>2</sub>O, and are probably very much less, especially at the cranial end of the thorax. The second difficulty of importance has been the peculiar nature of the thoracic inferior vena cava itself. Structurally, it is very largely non-cellular, as it consists for the most part of collagen and elastic fibres. It changes in length, as the diaphragm contracts, many thousands of times during the day. It has a scanty endowment of *vasa vasorum*. It is, therefore, a vessel *sui generis*, and in consequence it is not so surprising that the isolated vein deteriorates rapidly, and that diffusion often takes place through its walls, though the superior vena cava does not show corresponding permeability. The experiments on this point are being continued, and are only referred to here in order to explain why recording has had to be extremely rapid, once the vein has been removed from the body.

#### METHOD.

The volume variations of the isolated superior venæ cavæ and thoracic inferior venæ cavæ of six dogs were recorded by means of a special apparatus. The superior venæ cavæ were kept at their natural length, and were exposed rapidly to internal pressures of Ringer's solution varying from 0, 2, or 4 cm. to 30 cm. and back again. The inferior venæ cavæ were exposed to similar pressure changes from 4 or 6 cm. Ringer's solution to 30 cm. and back again, but this was done at both the expiratory and inspiratory lengths of the vessels, and usually also at intermediate lengths. The volume at each 2 cm. difference of pressure was automatically recorded by an optical device, and a complete cycle from low to high pressure and back took 2-4 seconds to record, so that the vein was exposed to the pressures for about the length of a normal respiratory cycle. Increase in internal pressure within the vein was assumed to be equal in its effect on the vein volume to a decrease in external pressure, such as would occur in the mediastinum of the intact animal.

The front and back views of the apparatus used are pictured in Figs. 1 and 2 respectively. A strip of teak *ab*, with another piece of teak *c* fastened on to it at right angles, is pivoted at *a* in a five-ply board *d*. The pivot is formed by one horizontal arm of a four-way metal tube *e*, which is firmly attached to the teak strip *ab*. Into the lower end of this metal tube is sealed a glass tube *f* of suitable calibre, which is held further down by the screw collar *g*. The lower part of the glass tube is bent round parallel to the teak strip *c* and has a flanged end. A second glass tube of

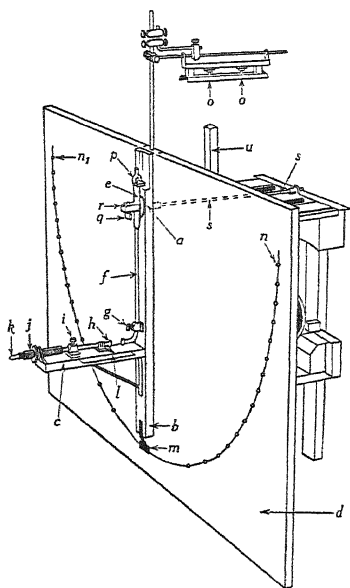


Fig. 1. Front view of apparatus described in text.

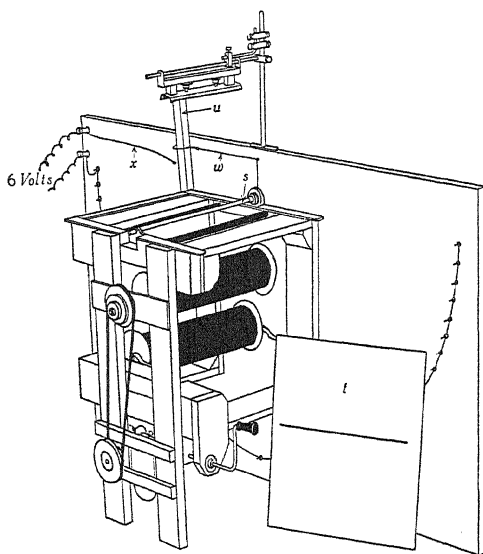


Fig. 2. Back view of apparatus described in text.

the same calibre, with one end *h* flanged and the other end *k* closed, is held in position by a screw collar *i* and can be moved backwards and forwards without rotation by a special device at *j*. An adjustable pointer on the glass tube *hk* indicates on a millimetre scale *l* the distance between the flanged ends of the glass tubes. A copper shoe *m* on the end of the teak strip *ab* is connected by a wire to the metal pivot tube, and makes contact with a series of thirty-one metal studs between *n* and *n*<sub>1</sub>. These studs are all wired together at the back of the board, and are so arranged that they flash on the 4 volt bulbs *oo* at 2 cm. pressure intervals as the shoe passes round the semicircle from *n* to *n*<sub>1</sub>. There are two taps on the four way

metal tube *e*; *p* is opened to let out air or fluid, *q* controls the socket *r*. The back view of the apparatus (Fig. 2) shows a detachable pipette *s*, which fits tightly, by means of an india rubber collar, into the pivot arm of the four way metal tube. The left-hand end of the pipette in the figure is open to the air. There are four rollers, shown in continuous black; the two lower ones are of wood with metal flanges, the two upper ones of brass. The gramophone motor indicated in the figure drives the upper wooden roller, and photographic paper (12 in. Kodak "Nikko" bromide soft) is thereby wound off the lower motor roller, and in its course passes over the left-hand brass roller, under the pipette, and over the right-hand brass roller onto the upper wooden roller. When records are being taken, the metal plate *t* is placed in position above the brass rollers and below the pipette, and the central slit in it lies immediately under the pipette. The lever *u*, when pushed to the left in Fig. 2, starts the motor and switches six volts into the lighting circuit, so that the lights flash on whenever the shoe *m* touches one of the metal studs in the semicircle *nn*<sub>1</sub>.

The isolated vein is tied over the flanged ends of the glass tubes and the tube-vein-tube-pipette system is filled through the free end of the pipette with Ringer's solution at appropriate temperature. While this is being done, tap *q* is kept shut and tap *p* is left open until all air has been expelled. Then tap *p* is shut, a syringe inserted into socket *r*, tap *q* opened, and the meniscus of fluid in the pipette adjusted to a suitable point by withdrawal of fluid into the syringe. Tap *q* is then shut and the syringe removed. Rotation of the teak strip *ab* round the semicircle from *n* to *n*<sub>1</sub> causes the vein to be exposed to internal pressures of Ringer's solution from 0 to 30 cm. and back again, and a photograph of the meniscus in the pipette is taken, at every 2 cm. pressure difference, upon the paper moving underneath the slit. The rotation of the teak strip is done by hand in red light in a thermostat room at 37° C.

## RESULTS.

*Dogs.* As the technique varied very little, one experiment only need be cited at length. First, the apparatus was put into the thermostat room. Then a dog weighing 14 kg. was tied down on its left side, and the normal extent of its respiratory movements noted. A subcutaneous injection of atropine sulphate was made, and thereafter anæsthesia was induced with ether and chloroform. The skin over a portion of the left hind limb was incised and a venous cannula inserted into a vein thus exposed. Nembutal "844" sterile solution, in the dosage of 1 c.c. per 5 lb. body weight, was

injected through the cannula over a period of eight minutes, the general anaesthetic being discontinued after the first two or three minutes of the injection. A tracheal cannula was next inserted, and artificial respiration was begun. The skin over the right thoracic wall was reflected and, during a temporary cessation of artificial respiration, a large portion of the wall was removed. Then artificial respiration was resumed for some minutes. In the meantime a clip was attached to the diaphragm by the side of the caval foramen; to this clip was fastened a string and to the string a rubber band, looped over a nail on a centimetre scale. When artificial respiration was again discontinued, the diaphragm resumed its contractions, and the excursion of the string-rubber junction was noted on the centimetre scale at a time when the respiratory movements were approximately as great as those previously observed in the intact animal. The excursion of the whole thoracic inferior vena cava, so determined, was about 15 mm. Two points on the surface of the vein were then marked lightly with a cautery, and the distance between them measured at the expiratory length of the vein; this was 44 mm. The length of the superior vena cava was also measured between two fixed points. The thoracic inferior vena cava was cut out of the body, and tied on between the glass tubes of the apparatus at less than its expiratory length. Ringer's solution was run in, the vein adjusted to its expiratory length, and the meniscus in the pipette brought well away from the free end with 30 cm. solution pressure inside the vein. A quick test was made without a photographic record being taken, and it was found that the volume of the vein at 44 mm. length and 4 cm. Ringer's solution pressure equalled the volume at 54 mm. length (moderate inspiration) and 20 cm. pressure. The length of the vein was again adjusted to 44 mm. and three photographic records were taken at pressures from 4 to 30 to 4 cm. Ringer's solution, and at lengths of 44 mm., 54 mm., and 57 mm., the last being the observed inspiratory length of this piece of the total vein. When the records had been taken, the volume of the vein at 57 mm. length and 30 cm. pressure was measured by filling the whole system, sealing the free end of the pipette, and withdrawing the fluid in the vein through the tap *g* (Fig. 1) by means of a syringe inserted into the socket *r*. This volume was 5.0 c.c. Then the superior vena cava was tied on at its normal length, and a record taken of its volume changes with pressures of Ringer's solution from 2 to 30 to 2 cm. Its volume, when measured at 30 cm. pressure, was 6.5 c.c., but the whole length of the vein was not used.

The results are shown graphically in Fig. 3. (The kink in curve I was due to the screw-collar at *i* being a little too slack, and was not due to the

vein in any way. Recording is best done rapidly, and it is not easy to alter the length quickly if this screw collar is too tight.) It seems probable, from the graph, that the volume of the thoracic inferior vena cava diminishes a little in inspiration. The curve for the superior vena cava was plotted from the averages of the double readings.

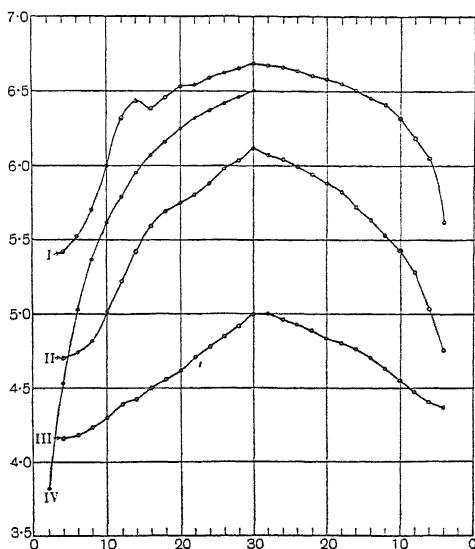


Fig. 3. Graph showing the volume of the isolated thoracic inferior vena cava of a dog at different lengths and pressures, and of the isolated superior vena cava of the same dog at different pressures. Curve I, inferior vena cava at expiratory length, 44 mm.; Curve II, at 54 mm.; Curve III, at 57 mm. (observed inspiratory length). Curve IV, superior vena cava. Ordinates: volumes in c.c. Abscissæ: pressures in cm. Ringer's solution.

The results with the veins from the other five dogs were similar in character to those already described. In the case of the thoracic inferior venæ cavæ, they were in brief:

(1) Volume at expiratory length, 42 mm., and 6 cm. pressure, was 5.7 c.c. Volume at inspiratory length, 52 mm., and 30 cm. pressure, was 5.3 c.c.

(2) Volume at expiratory length, 36 mm., and 6 cm. pressure, was equal to the volume at inspiratory length, 44 mm., and 30 cm. pressure.

(3) Diffusion was seen to be taking place through the wall of this vein during the experiment, but even so the recorded volume at expiratory length and 20 cm. pressure was equal to that at inspiratory length and 30 cm. pressure.

(4) Volume at expiratory length, 45 mm., and 4 cm. pressure, was greater by 14 p.c. than the volume at 53 mm. length and 30 cm. pressure.

(5) Volume at expiratory length, 43 mm., and 9.5 cm. pressure, equalled the volume at 53 mm. length and 30 cm. pressure.

From the intrapleural pressure records in the literature, one would gather that the changes in the intramediastinal pressure are likely to be much less than those necessary in the above experiments to equalize inspiratory and expiratory volumes, i.e. this vein in the dog probably decreases in volume in inspiration, if structure and intramediastinal pressure are the only two factors which are concerned.

*Cats.* The technique was similar, except that tubes with smaller flanges were used, no photographic records were taken, and only the thoracic inferior venæ cavæ were tested. The results were even more marked than the canine ones:

(1) Volume at expiratory length, 30 mm., and 4 cm. pressure, was 0.84 c.c. Volume at inspiratory length, 37 mm., and 30 cm. pressure, was 0.68 c.c.

(2) Volume at expiratory length, 30 mm., and 4 cm. pressure, was 0.8 c.c. Volume at inspiratory length, 40 mm., and 30 cm. pressure, was 0.72 c.c.

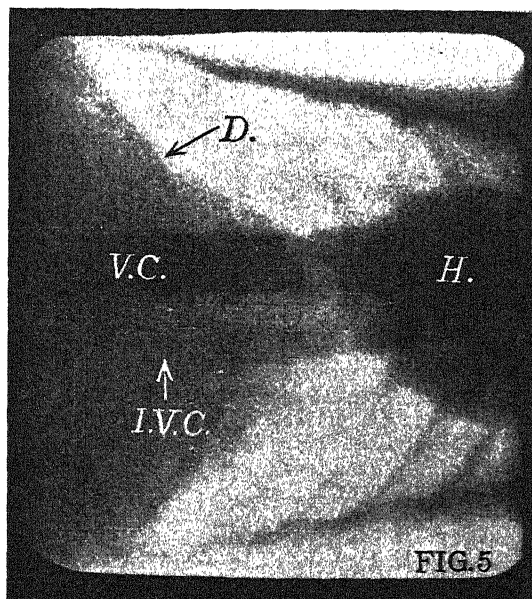
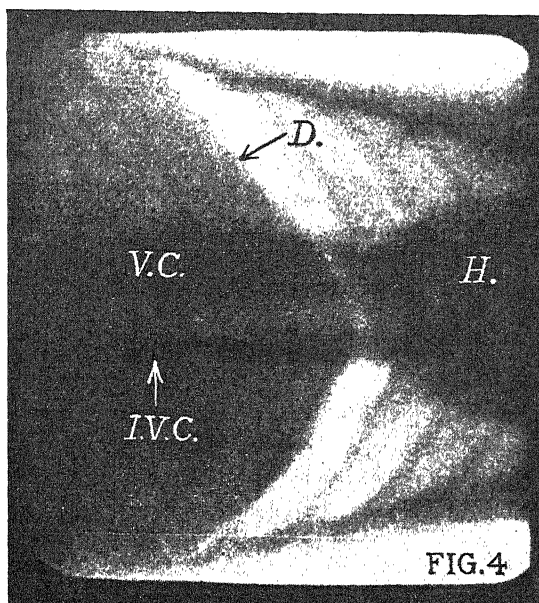
(3) Volume at expiratory length, 27 mm., and 4 cm. pressure, was 0.75 c.c. Volume at inspiratory length, 35 mm., and 30 cm. pressure, was 0.68 c.c.

*Rabbit.* Only one rabbit was used. The volume of its isolated thoracic inferior vena cava at expiratory length, 28 mm., and 4 cm. pressure, was 0.91 c.c., at inspiratory length, 33 mm., and 30 cm. pressure, was 0.87 c.c.

In all the dogs, and in at least one of the cats, there was a band of diaphragmatic muscle passing over the inferior vena cava at the caval foramen. This has been noted before [Franklin, 1933] as worthy of more careful investigation, for it might constrict the vein at this point in inspiration. In one of the dogs it certainly did so, but further work is necessary before any general statement can be made, and only this passing reference will be inserted here.

As a control to the experiments recorded above with the isolated vein, Dr Robert Janker (of the Röntgeninstitut der chirurgischen Universitäts—Klinik und Poliklinik, Bonn) kindly undertook to make for the writer an X-ray cinematograph film. X-ray opaque substance (Perabrodil or Thorotrast) was injected into the femoral veins of four cats and a record taken in each case of its progress along the inferior vena cava to the heart. In Figs. 4 and 5 a thoracic inferior vena cava is shown in expiration and inspiration respectively, and it is quite clear that the





Figs. 4, 5. X-ray cinematograph pictures showing the thorax of a cat in the expiratory (Fig. 4) and inspiratory (Fig. 5) phases of a respiratory cycle. X-ray opaque substance in the inferior vena cava, showing black in the photographs. *I.V.C.* Inferior vena cava. *V.C.* Vertebral column. *D.* Anterior edge of diaphragm shadow. *H.* Heart.

volume of the vein is less in the latter phase. From the results of the writer's work, it is certain that one of the factors causing this diminution in volume is the structural peculiarity of the vein and its lengthening in inspiration. When the film is projected, it leaves no doubt in the mind of a spectator about the correctness of the views stated above, and thereby tends also to justify the suggestions, made elsewhere in this paper, about the intramediastinal pressure variations.

#### DISCUSSION.

Haller [1756, 1760, 1762] was the first to investigate in detail the effects of respiration upon the veins and upon the movement of the blood within them. His general conclusion [1762, p. 300] was that in inspiration the blood was dammed back in the abdominal inferior vena cava and flowed in more freely from the superior vena cava, in expiration the opposite. In inspiration, also, owing to the increased volume of the vascular bed in the lungs, blood flowed more freely into these organs from the heart, and into the heart from the vena cava. It is to be remembered that in most of Haller's experiments no narcotic was used, and doubtless in many cases the respiration was excessive and dyspnoic. Under such circumstances the thoracic inferior vena cava would lengthen to its maximum in inspiration, and it is highly improbable that the great diminution in volume of the vessel, which its structure would tend to impose with this degree of lengthening [Franklin, 1931, 1932, 1933], would be counterbalanced by the effect of the decrease in intramediastinal pressure (Fig. 3). Haller found that the inferior vena cava between the kidneys and diaphragm emptied itself and collapsed in inspiration, while in expiration it filled up and became round. He also noted a back-wave of blood or of injected air in inspiration in this portion of the vein. Haller's results can be understood if the volume of the thoracic portion of the vein and its lumen become smaller in inspiration, for at the onset of this phase some of the blood in the vein would be pushed backwards towards the abdomen, some towards the heart, and thereafter the inflow would tend to be less through the narrowed tube. Hence, after an initial extra inflow of blood from this vein into the heart, the inflow from the superior vena cava would be easier and this vein might itself be increasing slightly in volume. In this connection Haller's experiment on a suckling pig [1756, p. 184] is of very great interest, because the respiratory oscillations in the tributaries of the superior vena cava continued in their entirety even after the chest had been opened on one side. This would rule out intramediastinal pressure changes.

Since the beginning of the nineteenth century [Barry, 1826] the conception of these pressure changes has gradually come into physiology, and theoretical considerations of their effects upon the intrathoracic veins have replaced anatomical and experimental investigation.

It is now suggested that the volume of the thoracic inferior vena cava decreases in inspiration in those mammals, in which this vein lengthens in inspiration, and has a particular structure [Franklin, 1931, 1932]. The dog, the cat, and the rabbit are among such mammals, the guinea-pig is not. But the guinea-pig breathes so rapidly that its fluctuations of intramediastinal pressure are probably very small, and in any case they can only act for fractions of a second, so the volume changes are probably insignificant.

The superior vena cava in most mammals will tend to increase slightly in volume and perhaps also in calibre in inspiration, but from all the evidence one would imagine that such increase is small.

In man the thoracic inferior vena cava is of small length and volume, and probably undergoes little variation in volume. Keith [1902], however, has suggested that the hepatic portion of this vein may be compressed during inspiration between the right crus of the diaphragm and the liver. If Keith's idea is correct, and one cannot accept in their entirety anatomical findings unchecked by physiological experiment, then diaphragmatic contraction would produce in man, by slightly different means, an effect similar to that which it produces in the dog, cat and rabbit. Keith's view would also explain Mosso's findings [1884].

On the whole, then, it would seem that respiration has little effect upon the total volume of the two intrathoracic venæ cavæ taken together. The great increase in blood volume within the thorax, which occurs in inspiration, must be due to increase in volume of the heart (Janker's films have shown that this dilates) and of the lungs.

#### SUMMARY.

Experiments on the isolated veins of the dog, cat, and rabbit, lead one to believe that the thoracic inferior vena cava decreases somewhat in volume in inspiration, and that the superior vena cava increases somewhat in volume in the same phase of respiration. That the volume of the cat's thoracic inferior vena cava does decrease in inspiration is shown by X-ray photographs taken by Dr Janker.

I wish to thank Prof. J. A. Gunn for the interest he has shown in this work; to our laboratory staff, especially to Mr Tuckey, I am indebted

for ready co-operation at all times. I also wish to acknowledge help given to me in the conduct of experiments by Mr B. B. Hickey, of University College, Oxford. The work has been made possible by the generosity of the Rockefeller Foundation, and forms part of a study of the venous return in mammals; I wish here to record my great gratitude to the Foundation for its support. Finally, I should like to thank Dr Janker for making the X-ray film of the cats, and Prof. I. de Burgh Daly for many references to work on intrathoracic pressures.

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EFFECT OF BREATHING ON THE INTRA-  
ABDOMINAL PRESSURE<sup>1</sup>.

By W. H. WILSON.

*(From the Department of Physiology, Faculty of Medicine, Cairo.)*

THE greater part of the work on this subject has been done in regard to the pathology of enteroptosis. Keith [1907], in an article on this condition, gives figures for the pelvic and intra-gastric pressures found by himself and other observers. In observations made in 1895<sup>2</sup> he found that in the erect posture the pressure just below the diaphragm varied in an adult man from +4 to +8 mm. Hg, the rectal pressure from +20 to +28. In the recumbent posture the intra-gastric pressure rose to 16 mm. Moritz [1895] found a rectal pressure of 28 mm., the part of this due to the superincumbent weight of the viscera being 20 mm., the part due to the tonus of the abdominal muscles (gastric P) 8 mm. Schwerdt [1896] observed a rectal pressure of 35 mm. of which 27 were due to gravity. These observers agree in finding a rise averaging 4 mm. during normal inspiration with a fall to the original pressure in expiration.

Some observers have found a negative intra-gastric pressure during deep inspiration. Keith ascribes this to the fact that their subjects suffered from the loss of abdominal muscular tone seen in enteroptosis in which condition the sub-diaphragmatic pressure may be constantly negative, this being increased by the expansion of the upper part of the abdomen in deep inspiration. Moritz found in twelve out of thirteen normal persons an increase in intra-gastric pressure proportional to the depth of inspiration. With maximal inspiration he observed that the pressure in the stomach rose to 35, in the rectum to 64 mm. Hg. With sustained inspiration this pressure fell slowly owing, he believed, to the gradual relaxation of the tone of the abdominal muscles. Both in normal and deep breathing the pressure fell at the end of inspiration to the base line, not rising again until the beginning of the next inspiration.

<sup>1</sup> This paper formed the subject of a communication at the meeting of the Society on July 1, 1933.

<sup>2</sup> See reference, Keith, A. 1923.

I have ventured to draw attention to this somewhat forgotten subject because the values quoted and those shown in the figure give an indication of the magnitude of the assistance given to the venous return in a healthy man by the abdominal pressure and the changes it undergoes in inspiration. In addition the graphic records given by Moritz [1895] and my own results support the view that expiration, at any rate in so far as the abdominal muscles are concerned, is a passive process of elastic recoil.

#### METHOD.

The observations recorded were made some years ago in the course of an investigation of the intra-pleural pressure by an indirect method. Two mercurial manometers were employed registering simultaneously, one the expiratory force (the intra-pulmonary pressure) in an expiratory effort starting from maximal or lesser degrees of inflation of the lungs, the other the corresponding intra-gastric (extra-pulmonary pressure). The reading of the second deducted from that of the first gives a figure which represents that factor in the expiratory pressure which is due to the elastic contraction of the inflated lungs, and which is therefore a measure of the intra-pleural negative pressure. In the investigation referred to the pleural pressure in three healthy adult men was found to be with maximal inspiration from  $-14$  to  $-16$  mm. Hg. (*Note.* Not yet published.) Through the mouthpiece to which the first manometer was attached passed a thin rubber tube 18 in. long, ending in a small rubber bag. External to the mouthpiece this tube was attached to the second manometer. The bag, which had a size of about 2.5 cm. by 1.5, when filled with air, was swallowed partially deflated and a sufficient quantity of air was afterwards injected through a T-piece on the manometer connection. The observations were made about 3 hours after a meal, swallowing being assisted by a small draught of water. Moritz employed a similar method with a somewhat larger bag and a water manometer.

The pressure changes in the intra-gastric pressure accompanying different types of breathing are illustrated in Fig. 1.

#### RESULTS.

Graph I in the figure shows a record of the two manometers, the upper (*B*) giving the expiratory force, the lower (*A*) the intra-gastric pressure. Before expiring into the mouthpiece the subject filled the lungs (not to the maximal extent). The effort was sustained for 20 sec. with an intra-pulmonary pressure of 43 mm. The corresponding intra-gastric

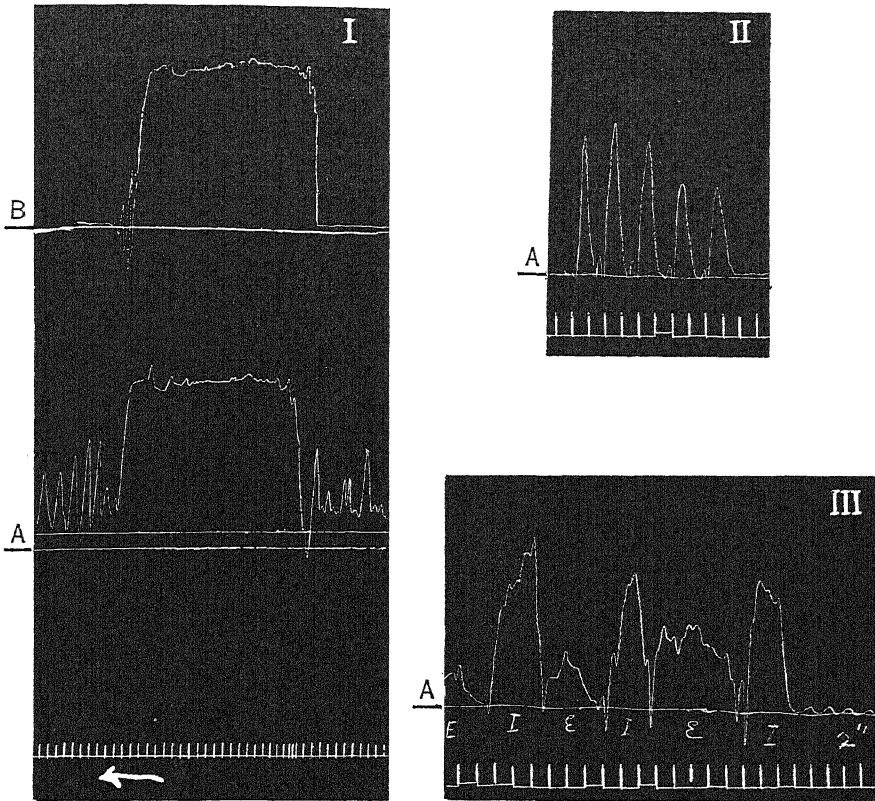


Fig. 1. Respiratory changes in intra-gastric pressure.

- I. Subject A. H. Posture: sitting erect. A. Intra-gastric pressure: rise of  $P=34$  mm. Hg. B. Intra-pulmonary pressure: rise of  $P=43$  mm. Hg. Intra-gastric  $P$  at base line  $A=6$  mm. Hg. To right of record on base line  $A$  pressure changes due to voluntary deep breathing. To left of record elevations of pressure accompanying hyperpnœa. Read right to left. Time record 1 sec.
- II. Subject D. N. Posture: sitting erect. Voluntary hyperpnœa. Rate of breathing 15 per min. Max. rise of  $P=40$ . A. Base line of intra-gastric pressure:  $P=5$  mm.
- III. Same subject and posture. Maximal inspiration followed by maximal voluntary expiration. A. Base line of intra-gastric pressure:  $P=5$  mm. Preliminary normal breathing (to right of record). Inspiration rise of  $P=3$  mm. Max. inspiration rise of  $P$  (3rd curve)  $=45$  mm. Max. expiration rise of  $P$  (1st curve)  $=23$  mm. Hg. I. Inspiration. E. Expiration. In both II and III breathing was unobstructed through mouth. Read right to left. Time record 2 sec.

pressure being 34 mm. This was preceded by a few fairly deep voluntary breaths and followed by rapid deep breathing, the dyspnoea resulting from the effort and absence of breathing. The effect on the intra-abdominal pressure is seen in the lower tracing, the rises corresponding to inspiration, the falls to expiration. The maximum rise of pressure accompanying the dyspnoea was 24 mm. Hg. The base line represents a pressure of about 6 mm. The fall below this, seen during the dyspnoeic hyperpnoea, is probably due to a relaxation of the abdominal muscular tone following the effort or resulting from the rapid breathing. The record of the upper manometer shows no indication of the breathing, as the mouthpiece was only in place during the expiratory effort.

Graph II shows the effect of voluntary deep breathing at a rate of 15 per min., no effort being made to fill the lungs completely. The rise of pressure in the stomach accompanies inspiration (max. 40 mm. in 4th breath) falling to the original base line when inspiration ceases. The intermediate slight rises are due to oscillations of the mercury.

In Graph III the subject was instructed to fill the lungs to their maximal extent, and follow this by a slow maximal expiration.

At the commencement of the record some small pressure changes due to normal breathing occur, a rise of 3 mm. with inspiration, falling to the base line in expiration. During the forced breathing it will be seen that in the third curve the intra-abdominal pressure reached 45 mm. In this case inspiration was maintained for 5 sec., the gradual fall of pressure during that period probably corresponds to the slow relaxation of tone of the abdominal muscles observed by Moritz under similar conditions. With cessation of inspiration the pressure falls to the base line to rise again with forced expiration, 23 mm. being reached in the fourth curve. In this observation, as in that shown in Graph II, breathing was unobstructed.

#### DISCUSSION.

From the records described above it is evident that, except in Graph III (and to a lesser extent in the first part of Graph I), where expiration is voluntarily carried beyond the normal limit, there is no such rise in the intra-abdominal pressure preceding inspiration such as would correspond to the active participation of the abdominal muscles in expiration. The results are, in fact, exactly those which would be expected if the abdominal walls were composed simply of an elastic material.

A further point of interest in connection with the form of the respiratory cycle appears in these records. It is shown in the pressure changes



accompanying normal breathing in Graph III, but is better seen in Moritz's observations with a water manometer [Moritz (1895), Fig. 5, p. 333], both in normal and deep breathing. Between the first phase of expiration and the succeeding inspiration the graph of intra-gastric pressure shows a horizontal line, indicating that in the cycle of diaphragmatic breathing a pause of some duration occurs forming the second phase of expiration. Gad and Head describe this pause as normal for the rabbit. M. Hammouda and I have described it as being present in the anæsthetized or decerebrate dog. Its occurrence in the normal human cycle has, however, been disputed [Schafer, 1932]. The intra-abdominal pressure changes suggest that the pause is normal in human diaphragmatic breathing.

The influence of the movements of respiration in aiding the return of blood to the heart is of course well recognized. In relation to the figures given, it is of interest to consider briefly the extent of this influence of the intra-abdominal pressure and the changes it undergoes in normal and deep inspiration, combined with the corresponding changes in the intra-thoracic pressure on the return of blood from the vena cava and the organs whose veins open directly into it. The following figures indicate the value of the assistance given in an adult man standing or sitting erect under conditions of (a) normal expiration, (b) normal inspiration, (c) maximal inspiration:

(a) Pelvic P	28 mm. Hg.	Thoracic P	-6	Combined effect	34 mm.
(b)    "	32    "	"	-8	"	40    "
(c)    "	68    "	"	-15	"	83    "

Converting the combined figures into centimetres of blood, (a) represents 43, (b) 51, and (c) 105. The following measurements may be compared. The average height of the dome of the diaphragm above the pelvic floor is given by Keith as 36 cm. The height of the right auricle (entrance) is about 40 and the highest point of the venous system (vertex) 83 cm. above the pelvic level. Muscular exertion may raise the abdominal pressure 30 mm. or, if the exertion is great (*e.g.* lifting or sustaining a heavy weight), considerably more. The value of the assistance given, against gravity, by the intra-abdominal P to the venous circulation is evident.

The chief abdominal organ affected by these pressure changes is the liver. It is clear that an increase of 45 mm. in the sub-diaphragmatic pressure must, by compressing that organ, greatly assist the outflow of blood into the hepatic veins and vena cava. The subject is possibly of more clinical than purely physiological interest. In conditions in which,

owing to a lack of tone in the abdominal muscles, a very low or negative sub-diaphragmatic pressure exists, it is easy to understand to what an extent the return of blood from the abdominal organs and the vena cava may be hampered.

#### SUMMARY.

1. There is a rise in intra-abdominal pressure during inspiration proportional to the depth of inspiration.
2. No rise of pressure occurs in normal expiration such as would occur if the abdominal muscles took an active part in that act.
3. In deep breathing the abdominal pressure may exceed the maximum blood-pressure in the vena cava due to gravity.

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## THE ELECTRICAL RESISTANCE OF STIMULATED MUSCLE.

By W. HARTREE.

*(From the Physiological Laboratory, Cambridge.)*

THE observations recorded here arose from an attempt to verify, or explain, certain anomalous results which appeared in the analyses of the initial heat production of stimulated muscle. In some of these the usual clear division of the initial heat into two phases, corresponding respectively to contraction and relaxation, did not occur, but subsidiary waves or other anomalies were present [see Hartree, 1931]. Earlier experiments were all at 0° C., but further cases of the same kind have recently occurred at room temperature, in the heat analyses to be described in the subsequent paper, using the very rapid Moll microgalvanometer and the quickest thermopile available. It seemed possible that the irregularities found might be due to changes in the thermal contact between muscle and thermopile during the alterations of tension associated with contraction. Moreover, even when the muscle was held as rigidly isometric as possible, small movements would undoubtedly occur, and these might change the flow of heat from muscle to thermopile and so affect the results of the analysis.

A possible test for the accuracy of the analyses, and a means of finding out whether such disturbances did in fact occur, was suggested by Prof. A. V. Hill as follows: Record the thermopile-galvanometer deflection curve (1) on stimulation, (2) on heating by a known amount with a high frequency current producing no stimulus, (3) on stimulating and heating simultaneously. If contraction affected the flow of heat from muscle to thermopile, then the difference between (3) and (1), which is the result of heating by the high-frequency current during contraction, should be different from (2), which is due to heating by the high-frequency current at rest. If the curve obtained by subtracting curve (1) from curve (3) were not the same as curve (2), this could be attributed to the same heating

having a different effect on the thermopile when the muscle was stimulated and when it was at rest, owing to change of shape, change of position, or change in degree of thermal contact.

It appeared immediately that the difference between curve (3) and curve (1) not only differed from curve (2) in shape but was also considerably smaller (about 8 p.c.). The latter result suggested the possibility that the resistance of the muscle was altered by stimulation, and this might be due, at least in part, to a change in the amount of muscle between the electrodes during contraction. It might, however, be caused by a genuine change in the electrical conductivity of the muscle during activity, due to one or other of the physico-chemical changes associated with contraction. The latter possibility was important enough to require further investigation.

The heating current employed was produced by the valve oscillator referred to in a previous paper [Hartree, 1932]; the frequency employed was about 100,000 cycles per second; the strength of the current  $C$  was measured by a vacuo-junction supplied by the Cambridge Instrument Company. The hot wire of the vacuo-junction was in series with the muscle: its thermocouple was connected either to the Moll galvanometer or to the moving magnet galvanometer usually employed. On stimulation  $C^2$  was always greater (for the same oscillator volts) by about 7 p.c., and since  $C^2 M$  (where  $M$  is the muscle resistance) was 8 p.c. less, it appeared that the resistance between the electrodes fell about 15 p.c. on stimulation.

A simple way of observing the effect without measuring it was to apply a steady heating current of appropriate strength and, when the deflection of the vacuo-junction had settled, to stimulate the muscle. This resulted in an appreciable increase in the deflection measuring the current in the vacuo-junction, which soon disappeared when the stimulus was over.

It was observed, however, that when the muscle was stimulated, although the ends were fixed as definitely as possible at the bone and the tendon respectively, there was yet a very appreciable motion in the muscle due to the thicker part near the bone extending the thinner part. The actual motion, of the surface at least, can be seen by placing some very small pieces of thread on the muscle and watching them when the muscle is stimulated. The motion of the part several millimetres from the bone end is inappreciable, but that of the thin end, which may extend 6–8 mm. above the top edge of the thermopile, is considerable. This may result in as much as 2 mm. of the muscle passing on to the thermopile during contraction and rather less passing the top electrodes, but quite a

sufficient amount to diminish the resistance between the electrodes considerably.

The muscle, a frog's sartorius (English *R. temp.*), was therefore set up in the reverse position, the bone being at the top and held by the wire to the tension lever. The lower electrode was near the bottom edge of the thermopile, and the tendon held at 2 mm. below this served to avoid having a very thin part of the muscle at this electrode. There was certainly less change than before in the amount of muscle between the electrodes and on the thermopile during contraction. The motion past the top electrode, though some distance from the bone, was inappreciable, and that past the bottom electrode was much less than before, as there was so much less thin muscle beyond the electrode to be extended. Even in this case, however, it is quite appreciable, and readings by thermopile and vacuo-junction taken as before showed an apparent fall of resistance of 7 or 8 p.c., instead of 15 p.c., on stimulation.

Analyses of the initial heat with the muscle in this position are smoother and more regular than previously, which probably shows that there is less motion of the part of the muscle on the thermopile than there was when it was set up in the usual position.

The galvanometer sensitivity was now increased so that the stimulating current only, when passed through the vacuo-junction for 1 sec., produced a good deflection (about 100 mm.), and photographic records of the deflection were made when stimulating the live muscle for 1 sec., when passing the same current through the muscle when dead for 1 sec., and also when passing a constant current through a carbon resistance for 1 sec. The three deflection curves, when reduced to the same maximum, were so similar that there was no doubt that there is little change, if any, of the stimulating current during the stimulus, so there can be little change of the resistance of the muscle during contraction. This method of course will not show if there is a sudden change of resistance immediately on stimulation.

The deflection curve can be analysed to give the current at different times during stimulation, using for the control a curve obtained by passing a current for  $1/20$  sec. through the vacuo-junction. Such analyses, however, are hardly necessary.

Lastly, a comparatively large steady heating current from the valve oscillator was sent through the muscle and the vacuo-junction, giving a deflection of 1200–1500 mm. The spot of light was brought back to the scale, and a stimulus given with a view to investigating the change of the heating current during stimulation. With the sensitivity used the stimu-

lating current itself, in parallel with the oscillator heating current, had a considerable effect on the vacuo-junction reading. To avoid this it was necessary to stimulate by the nerve, and in order to eliminate as far as possible any effect due to the motion of the muscle past the electrodes, Prof. A. V. Hill kindly supplied an insulated frame with two rigid silver electrodes suitably placed, to which the muscle could be firmly tied. This frame was equipped with a pair of stimulating electrodes, on which the nerve to the sartorius could be laid.

Ten experiments were made with this arrangement, and the results were quite conclusive in showing that when movement is prohibited as far as possible there is no measurable change in the electrical resistance of the muscle during contraction. The extra deflection on stimulation for 1 sec. varied between  $-10$  and  $+9$ , the mean being about  $-1$ . The total deflection was about 1400, so that the electrical resistance was unaltered, on the average to within about 1 part in 1000 during contraction. All results to the contrary are presumably due to movement, change of shape, or similar disturbing factors.

Even when the muscle was tied firmly to the electrodes and there could be little or no motion past them, it was obvious that there was still a change of shape of the part of the muscle between the electrodes, due to the extension of the thinner end by the contraction of the thicker. The maximum motion observed was about 1 mm. Such change of shape must necessarily result in a slight increase of resistance, which no doubt accounts for the negative change (*i.e.* diminished heating) observed in some cases. As regards the positive results, it could be seen that the motion of the muscle past the electrode near the thicker end was very small, even when untied: the motion, however, past the electrode near the thinner end was always in the direction of the thicker end and might be quite large if the muscle were not tied there: thus the effect of any motion past the electrodes due to insecure tying up (and it is impossible to tie very tightly owing to danger of injury) is such as to increase the amount of muscle between the electrodes, and so to diminish its resistance and increase the heating.

Since the maximum effect of contraction on the resistance is less than 1 p.c., and since the mean is less than errors of observation, it seems clear that there is no real change of resistance during contraction.

It is not easy to reconcile the above results with two papers by McClendon [1912, 1927]. In his second paper McClendon found that the impedance of a muscle to a current of 1000 cycles was very much greater when the muscle was at rest than during stimulation. The method

was to place the muscle between two platinum plates and to determine the conductivity (*a*) when the current passing between the plates was too small to excite, and (*b*) when it was large enough to produce contraction. It is possible that the difference between the present results and McClendon's is due to the fact that he employed an alternating current of relatively low frequency, so that the impedance he measured may have been due in part to the impermeability of membranes. With the high-frequency current employed in the present experiments the membranes can have produced very little effect. It is not safe, however, to assume that conductivity is the same with a large current as with a small one, and this and other reasons may have led to McClendon's result. It is impossible, in view of the present results, to suppose that any considerable change in the real conductivity of the muscle can have occurred during contraction.

#### SUMMARY.

When the movement of a muscle past the electrodes is prevented, its electrical resistance to a current of 100,000 cycles per sec. is not measurably affected by stimulation. The physico-chemical changes, therefore, during contraction are not such as to produce a measurable increase in the number of ions free in the muscle fluid, or in their mobility.

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## A REVISED ANALYSIS OF THE INITIAL HEAT PRODUCTION OF MUSCLE.

By W. HARTREE.

(*From the Physiological Laboratory, Cambridge.*)

WHEN a frog's sartorius muscle is mounted on a thermopile in the usual way, with a considerable length of the narrow part of it beyond the end of the thermopile, it can be seen on stimulation that there is an appreciable motion of muscle on to the thermopile due to the extension of the thinner part by the contraction of the thicker. This may happen although the ends of the muscle may be quite rigidly fixed. It is possible that this movement may have affected previous heat analyses to some extent, so revised analyses have been made, mounting the muscle in the reverse direction. The lower electrode is placed quite close to the end of the thermopile, and with the new arrangement the thinner end of the muscle is taken only 1 or 2 mm. beyond this electrode and is held firmly by the tendon and tied round fairly tightly at the electrode. Thus, during contraction, the motion of muscle on to the thermopile at this end is very small. At the other end of the thermopile the motion of the muscle on stimulation is negligible. Thus, with this method of mounting the muscle there can be very little change during contraction in the amount of muscle on the thermopile: its shape certainly alters perceptibly but this is unavoidable, and it can only be hoped that this change in shape is not sufficient to have such an effect on the flow of heat from muscle to thermopile as to upset analyses made by means of control heating curves taken when the muscle is in its resting shape.

### RESULTS.

#### A. *Twitches.*

The results of the analyses in several experiments on single twitches at 0° C. were at least fairly uniform. Two curves are given in Figs. 1 and 2, these being somewhat similar to Fig. 5 in a previous paper [Hartree, 1931]. Since no cases like the anomalous figures with multiple "humps," or drawn out initial outbursts (Figs. 6 and 7 in that paper), were found,



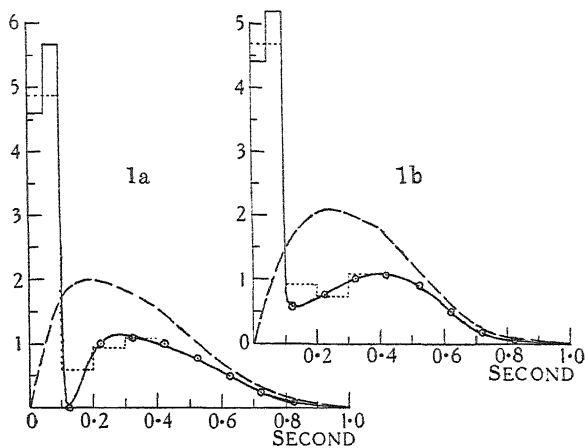


Fig. 1.

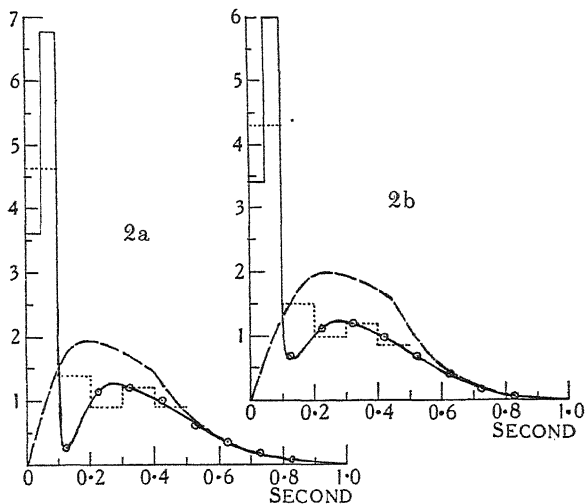


Fig. 2.

Figs. 1, 2. Single isometric twitches of frog's sartorius (English *R. temp.*) at  $0^{\circ}\text{C}$ . Full lines: results of heat analyses using control with 0.05 sec. heating and starting with two steps of 0.05 sec. Dotted lines: results of analyses of the same curves using control with 0.1 sec. heating (for comparison see text). Broken lines for tension curves: these show in every case a distinct "shoulder" between 0.4 and 0.45 sec., presumably due to friction between muscles and upper electrode. The tension curve ends very closely with the heat curve, between 1.0 and 1.2 sec., but cannot be shown clearly beyond 1 sec. Fig. 1 refers to one pair of muscles and Fig. 2 to another pair, these two experiments being chosen to show the greatest observed difference before 0.1 sec. Cases *a* refer to early stimuli and cases *b* to later and somewhat stronger stimuli.

The unit for the vertical scale is the total initial heat per sec. for each case.

it must be admitted that the peculiarities there shown were probably due to motion of the muscle on, or on to, the thermopile affecting the flow of heat from one to the other.

The course of the heat production during a twitch occurs in two phases, as has always been found before. If we regard the mechanical response as due to the formation and decay of some substance, or to the development and disappearance of some physico-chemical state, it is natural to associate one phase of the heat with the formation or development, the other with the decay or disappearance. Regarding the matter in another way, the first phase is waste heat in the development of tension, the second is potential energy degraded as the contraction disappears. It is possible, however (though see the discussion of Fig. 3 below), that the "dip" at 0.1 sec. may possibly be due either (*a*) to motion of the muscle during the rise of tension, or (*b*) to part of the muscle being less active than the rest.

Every precaution was taken to reduce the effect of motion of the muscle, and in the experiments referred to it is believed that the change in the amount of muscle on the thermopile during contraction was negligible; there is still the possibility, however, of an effect due to the change of shape, and this cannot be avoided.

As regards (*b*) it was noticed that for a single twitch the maximum tension seemed to increase continually as the strength of the stimulus was increased; thus, although care was taken to give a fairly strong stimulus it was always possible that there was some small region of inactivity in the muscle, and if the less active part were not in contact with the thermopile the result of such inactivity would necessarily show as a fall in the heat rate, *i.e.* of heat reaching the thermopile, quite soon after the stimulus. This possibility is discussed in another connection in a recent paper [Hartree, 1932]. It seems rather unlikely, however, that the inactive region would occur so regularly in the same position relative to the thermopile as always to give an artificial appearance of a separation between the contraction and relaxation phases of the heat production: though it must be admitted that the same side of the dissected muscle was always outside.

Without further improvement in technique, however, by which change of shape is altogether prohibited, or its possible effect annulled, it does not seem possible to obtain more decisive results.

A few details of the analyses should be mentioned. In every case control heating curves were taken by passing a high-frequency current through the living muscle for 0.05 sec.: this did not stimulate it. When, as in Fig. 2, the heat produced during the first 0.1 sec. is far from

uniform, it is necessary, in order to avoid an early large remainder in the analysis, to start with two steps of 0.05 sec., but there is no advantage in continuing with steps of less than 0.1 sec. From the above control it is easy to construct one for 0.1 sec. heating, and the result of using this is shown by the dotted line; it is clear that the use of such a control in cases in which the heat rate is varying rapidly does not give a good estimate of the heat occurring in each 0.1 sec., and the estimate of that appearing between 0.1 and 0.2 sec. is so wrong as to be very deceptive.

It is obvious that, when using the 0.1 sec. control, any heat really occurring at 0.1 sec. will be considered as half from 0 to 0.1 and half from 0.1 to 0.2 sec. Similarly any heat really occurring uniformly between 0.05 and 0.1 sec. will be considered as  $\frac{3}{4}$  from 0 to 0.1 and  $\frac{1}{4}$  from 0.1 to 0.2. In Fig. 2 there is large heat from 0.05 to 0.1 sec. (not necessarily uniform), consequently the analysis by 0.1 sec. heating control will necessarily show about a quarter of this between 0.1 and 0.2, although in fact there may be no real heat in that interval. A further necessary consequence of taking too much heat in the interval 0.1 to 0.2 is that (to keep the remainders small) too little must be taken in the next step 0.2 to 0.3 sec., etc., the dotted line "oscillating" on each side of the more correct full line, the results being indistinguishable after 0.4 sec. It happens that the dotted lines avoid the "dip" at 0.1 sec. and so perhaps give a more reasonable looking result than the full line, but there is no doubt that the full line gives the best result possible from the observations.

The records for the twitch of Fig. 2a and the corresponding heating control are given for comparison with the cases which follow.

Sec. ...	...	0	0.05	0.1		0.2		0.3		0.4		0.5		0.6		0.7		0.8
0.05 sec. control	0	5	50	152	289	434	564	680	776	848	901	939	963	980	991	995	998	999
Fig. 2a	0		10		106		263		428		578		709		813			890
Heat	0.180	.34	0.03		0.115		0.12		0.10		0.06		0.035		0.022			
Sec. ...	...	0.9		1.0		1.1		1.2		1.3		1.4		1.5		1.6		
0.05 sec. control	1000	999	998	997	995	994	992	990	988	986	984	982	980	978	976			
Fig. 2a		943		975		988		996		999		1000		999		998		
Heat				0.01 approx.														

Details of the thermopile are given in the appendix. A Downing moving magnet galvanometer was used, sensitivity 1 mm. at 1 m. =  $6.5 \times 10^{-9}$  amp., critically damped, total period undamped 0.81 sec., resistance  $12\frac{1}{2}$  ohms.

### B. Tetanus.

A number of experiments were made using the Moll microgalvanometer constructed by Kipp. Although this galvanometer is not sufficiently sensitive to give good sized records for a single twitch, it can be used with excellent results for a tetanus. At 0° C. a tetanus of 0.5 to 1.0 sec. would give a deflection of about 50 mm., and at room temperature a tetanus of 0.2 to 0.3 sec. a deflection of about 100 mm. The mirror of the galvanometer is comparatively large, so good photographic records can be taken at a distance of 4 m. and the spot is always very steady. The action of the galvanometer is so rapid (total period 0.193 sec.), and the thermopile is so quick, that definite analyses can be made using steps of only 0.05 sec. This is necessary for experiments at room temperature (see below), but the galvanometer was also used for a few cases at 0° C., the

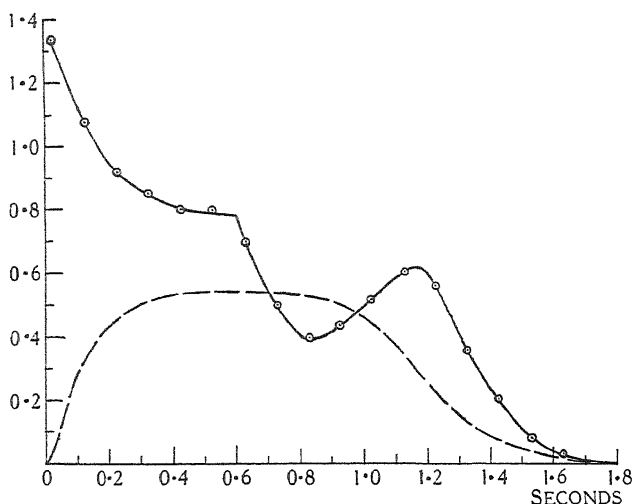


Fig. 3. Full line: result of heat analysis for 0.6 sec. tetanus at 0° C., employing the Moll galvanometer. Broken line for tension. The curves appear to end together. The numbers on the vertical scale must be divided by 1.02 to express heat rates in terms of the total initial heat per second.

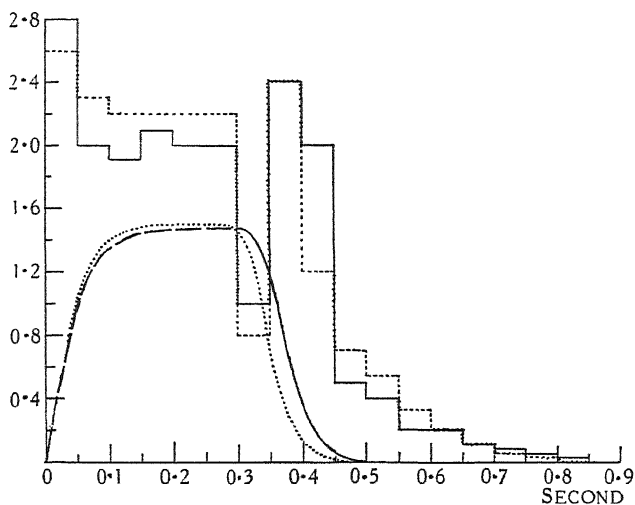


Fig. 4. Results of heat analysis for stimulus of 0.3 sec. approximately, at 17° C. Full line: result of heat analysis using Moll galvanometer and steps of 0.05 sec. Broken line for tension. Dotted lines for an experiment on another day, with stimulus about 0.28 sec., showing very similar results. In each case the heat production lasts much longer than the tension, so that only the early part of the heat production after the stimulus can be associated with the fall of tension energy. The vertical scale gives the heat rate in terms of the total initial heat per second.

result being always very "smooth," as in Fig. 3, of which the observed curves were as follows:

Sec. ...	...	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0.05 sec. control		0	320	833	952	981	993	999	1000	999	998
0.6 sec. stimulus		0	16	73	125	169	213	253	292	324	349
Heat	...	0.067	0.054	0.046	0.043	0.040	0.040	0.035	0.025	0.020	0.022
Sec. ...	...	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	
0.05 sec. control		995	991	987	983	978	973	968	963	957	
0.6 sec. stimulus		372	393	421	448	472	488	496	499	500	
Heat	...	0.026	0.030	0.028	0.018	0.010	0.004	0.002			

Details of the thermopile are given in the Appendix. The Moll micro-galvanometer had a sensitivity of 1 mm. at 1 m. =  $4 \times 10^{-8}$  amp.; it was critically damped and had a resistance of 21 ohms.

It is clear, in this case at least, that the sudden drop in the rate of heat production at the end of a stimulus cannot be due to motion of the muscle, since in Fig. 3 the tension did not begin to fall appreciably for some time afterwards; indeed the rate of heat production had reached a minimum before any movement could have occurred. This is pertinent to the question, discussed above for the case of single twitches, of whether the break in the heat production just after 0.1 sec. might be due to movement.

In this, as in all the following cases, the muscle was mounted in the "reverse" position as described for the case of a single twitch. Usually, after the records had been taken, the muscle was observed when stimulated; its motion past the top end of the thermopile was always negligible and that at the bottom end of the thermopile was usually of the order of 0.5 mm.

The analyses at room temperature (16°–17° C.) agreed well. Several different experiments with stimulus 0.3 sec. gave very similar results, of which two are shown together in Fig. 4. The observed curves for stimulus and control, in the case of the experiments corresponding to the full line, were:

Sec. ...	...	0	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45
0.05 sec. control		0	60	359	670	838	914	946	964	976	985
0.3 sec. stimulus		0	8	57	136	224	320	417	512	595	683
Heat	...	0.14	0.10	0.095	0.105	0.10	0.10	0.05	0.12	0.10	0.025
Sec. ...	...	0.5	0.55	0.6	0.65	0.7	0.8	0.9	1.0	1.1	1.2
0.05 sec. control		991	995	997	999	1000	1000	999	998	996	993
0.3 sec. stimulus		778	849	896	927	946	973	988	994	998	1000
Heat	...	0.02	0.01	0.01	0.006	0.005	0.004	0.003			

Thermopile and Moll galvanometer as before.

In every case the heat went on very appreciably after the tension had fallen to zero, which must be due to genuine delayed heat, as described

in a recent paper [Hartree, 1932]. It cannot be attributed to delayed appearance of heat, due to inactivity of some of the muscle, as it comes too late for that and is about the same in every case. The early part of the heat production was very regular, in fact it is remarkable how steady was the heat rate during stimulation after the first 0.05 sec.

The analyses with steps of 0.05 sec. were as good and as definite as in the previous case of a single twitch with steps of 0.1 sec. By comparing the tables above it can be seen that the 0.05 sec. control with the Moll galvanometer is now higher at 0.1 sec. than it was with the slower moving magnet galvanometer at 0.2 sec. The analysis would be even better using a control employing still shorter heating, but with the high frequency oscillator available the amount of heat, and therefore the shortness of the heating, is limited. The improvement is entirely due to the quicker galvanometer action since the same thermopile was used in every case.

The Downing moving-magnet galvanometer could not take advantage of the comparatively large amount of heat in the tetanus at a higher temperature since, if it were made very insensitive and very rapid, the damping could not be increased sufficiently. It was necessary, therefore, to increase the damping as much as possible and then to diminish the sensitivity with a resistance.

#### SUMMARY.

It seemed possible that the analysis of the heat production during the initial phase of muscular contraction might be influenced by slight movements or changes of shape of the muscle during contraction. A modified arrangement has made it possible to eliminate to a considerable degree the effect of such possible disturbances. When this is done the anomalous cases described in an earlier paper disappear and the heat production in a single twitch is found to occur in two phases only—contraction and relaxation. In a tetanus the same two phases occur, and so long as the stimulus is continued the rate of heat production persists at a nearly constant but slightly decreasing rate, following a more or less sharp fall soon after the beginning of the stimulus.

I am greatly indebted to Prof. A. V. Hill for his encouragement and advice.

The Moll microgalvanometer referred to in this paper was purchased with a grant from the Royal Society.

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## APPENDIX.

*The properties of the thermopile employed.*

The instrumental factors involved in the attainment of the greatest possible sharpness of analysis have been discussed by A. V. Hill (*Adventures in Biophysics*, Philadelphia, 1931, Appendix I, p. 142).

The properties of the galvanometers used in the present investigation have been given in the text: those of the thermopile are as follows (a single instrument, constructed by Mr A. C. Downing, was used throughout):

67 flat constantan-iron couples in a distance of 14 mm., resistance 23.7 ohms, bakelite insulation.

*Quickness of response.* This was determined by analysing a 0.05 sec. heating record by means of the galvanometer deflection due to an impulsive current. The latter might have been obtained experimentally: it was simpler and as accurate, however, to calculate it from the equation

$$y = \alpha t e^{-\alpha t},$$

where  $\alpha = 2\pi/T$ ,  $T$  being the complete period (= 0.193 sec.). With this the current in the thermopile, during and after 0.05 sec. heating, had the following relative values:

Sec. ...	0.025	0.05	0.075	0.10	0.125	0.150	0.175
Current	19	45	68	83	89	93	95
Sec. ...	0.200	0.225	0.250	0.275	0.300	0.325	
Current	97	98	99	99½	100	100	

For instantaneous heating half the maximum thermopile current would be developed in about 0.04 sec., 95 p.c. of it in about 0.14 sec. The Moll galvanometer attains 95 p.c. of its full deflection to a constant current in about 0.10 sec., so is slightly faster than the thermopile.

## THE EFFECT OF IONS ON THE CUTANEOUS SENSORY ENDINGS OF THE FROG.

By M. TALAAT.

*(From the Physiological Laboratory, Cambridge.)*

THE effect of various ions on the response of sensory endings has been studied by Matthews [1931] in the case of the frog's muscle spindle. The results agreed with the known effects of ions on excitable tissues, for absence of  $\text{Ca}^{++}$  was found to increase the excitability and prolong the discharge of nerve impulses and excess of  $\text{K}^+$  to depress it.

Feng [1933] has shown the depressant effect of increased  $\text{K}^+$  on the sensory endings in the skin, but there has been no systematic work on the behaviour of cutaneous endings in solutions with diminished  $\text{Ca}^{++}$  content. The point seemed worth investigating because the cutaneous endings in the frog are mainly of the type which becomes very rapidly adapted to a constant stimulus, whereas muscle spindles adapt slowly. Adrian and Gelfan's recent work [1933] on muscle fibres has emphasized the common basis of rhythmic impulse discharges, and it was hoped that further study of sensory endings might also be of value from this point of view.

### METHOD.

An isolated piece of frog's skin with its nerve supply formed the material for most of the experiments. The skin was usually slightly stretched and pinned at the edges to the floor of a small wax chamber that held about 2 c.c. of fluid. In a few experiments the antidromic preparation devised by Adrian, Cattell and Hoagland [1931] was used. The cutaneous nerve was placed on two silver-silver-chloride electrodes, and the nerve endings were stimulated by touching the skin with a glass rod or by application of a 5 g. weight.

The action currents from the nerve were photographed with a Matthews' oscillograph and viewed in a revolving mirror. Observation of the discharge by a loud-speaker system has proved of great value in many of the lengthy experiments.



The chief solutions used were:

		p.c.		p.c.
(1) Ringer	NaCl	0.65	NaHCO <sub>3</sub>	0.015
	KCl	0.02	CaCl <sub>2</sub>	0.025
(2) Ca and K free Ringer	NaCl	0.695	NaHCO <sub>3</sub>	0.015
	NaCl	0.675	NaHCO <sub>3</sub>	0.015
(3) Ca free Ringer	KCl	0.02		
	NaCl	0.67	NaHCO <sub>3</sub>	0.015
(4) K free Ringer	CaCl <sub>2</sub>	0.025		

The osmotic pressures of these solutions differ very little and the anion content is practically the same. As was shown by Matthews [1931], any difference in the behaviour of the end organs when bathed with such solutions must be attributed to the metallic ions.

Other solutions used will be referred to later.

### RESULTS.

*Absence of Ca and K.* When the isolated skin preparation is kept in Ringer at room temperature (12–19° C.) no resting discharge can be detected in its nerve supply, unless the area supplied by the nerve has been cut through during the dissection when slow impulses of small magnitude appear. [Cf. Adrian, 1931.]

If the Ringer is replaced by a K and Ca free solution, it is found that, within 5 min., the end organ gives for the same stimulus (a 5 g. weight) a greater discharge than in Ringer (Fig. 1). The frequency of the impulses is increased at the moment when the weight touches the skin, and the discharge continues for a few seconds instead of subsiding almost at once when the weight comes to rest on the skin. This effect increases steadily as the skin remains in the solution, the discharge subsiding more and more slowly under a constant stimulus.

A stage is reached at which the discharge resembles that of a muscle spindle in that it is maintained until the moment when the stimulus is removed. This is followed (20–30 min. after the application of the solution) by a stage in which the discharge outlasts the stimulus. At first the after-discharge is of low frequency and lasts only for several seconds, but after the skin has been in the solution for about 1½ hours it lasts from 3 to 10 min. and is of high frequency. By touching the skin with a fine pointed glass rod it is possible to arouse the activity of single end organs and to study the evolution of this discharge. It begins with a frequency above 100 per sec. and is at first regular (Fig. 2 A). The frequency declines steadily (Fig. 2 B) and the discharge becomes quite irregular when it has fallen below about 25 per sec. But in many

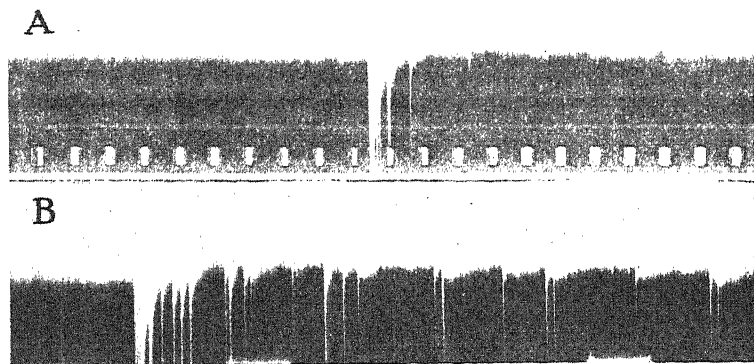


Fig. 1. Sensory impulses in dorsal cutaneous nerve of frog. The effect of dropping a load of 5 gm. on the skin while (A) in Ringer, and (B) 1 min. after application of NaCl,  $\text{NaHCO}_3$  solution. In both cases the load was left till the end of the record. Time marks (white strips in B) = 0.6 sec.

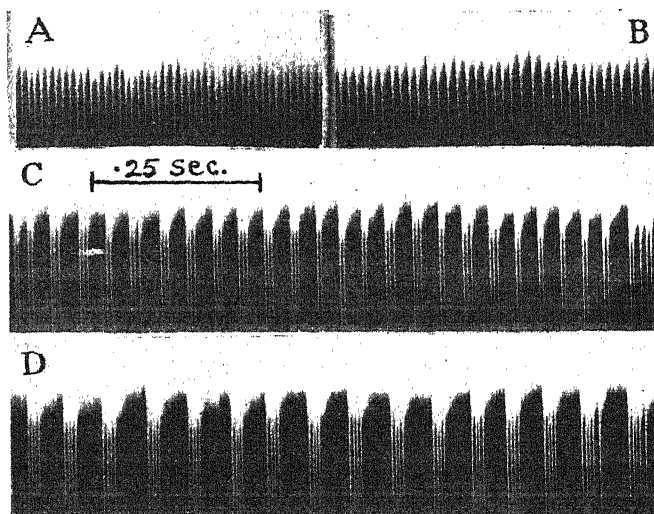


Fig. 2. After-discharge developed in NaCl,  $\text{NaHCO}_3$  solution, skin touched with a fine pointed glass rod. (A) 30 sec., and (B) 60 sec. after removal of the stimulus. (C and D) rhythmic grouping occurring in another ending 3 min. after the stimulus (touch). All records at same speed.

preparations the decline in frequency is associated with a well-marked grouping of the impulses, of the same type as that found by Adrian [1930] in injured mammalian nerves and by Matthews in the frog's muscle spindle in NaCl [1931]. The grouping is shown in Fig. 2 C, D. The groups usually develop as they do in mammalian nerves. The number of impulses in the group increases gradually from two to five or more and, as the number increases, the interval between the first impulses in the group becomes shorter and shorter, so that eventually the second impulse may fall within the relative refractory period of the first [cf. Adrian, 1930, Figs. 4, 5]. As in mammalian nerves a definite increase in the number in the group is preceded by a stage in which the number fluctuates between the lower and the higher value.

In addition to this increased activity on stimulation, some of the end organs begin to show a resting discharge, but usually this is of low frequency and is irregular.

The preparation recovers readily if put again in Ringer's solution.

*Absence of Ca.* When the fluid contains 0.02 p.c. KCl in addition to the NaCl the discharge becomes prolonged in the same way but the frequencies are lower than in NaCl alone. The discharge which occurs as the immediate response to a stimulus is, if anything, slightly less than in ordinary Ringer. The slowing of adaptation is shown by the development of a resting discharge (the skin being under slight tension) and usually a stage is reached at which a touch gives a long after-discharge like that in NaCl. The maximum frequency (60 per sec.) is not so high; grouping of the impulses may occur but the spacing of the impulses in the group is wider than in NaCl (30–50 per sec.).

The absence of Ca does not seem to be detrimental to these end organs, as they are still excitable after a period of 5 or 6 hours in this solution.

*Absence of K.* When the fluid contains Ca, but not K, the response of the end organ does not differ appreciably from that in Ringer except that a slow resting discharge may appear. No after-discharge was noticed in any of the eleven experiments made.

*Absence of electrolytes.* It may be mentioned that, if the skin is immersed in isotonic cane-sugar solution, the excitability of the end organs disappears in about 1–1½ hours. On replacing the sugar by the NaCl, NaHCO<sub>3</sub> solution the excitability returns to some of the end organs within a minute and to practically all of them within 10–15 min. This suppression of activity and recovery can be repeated several times without apparent harm to the end organs.

*Solutions containing oxalate or citrate.* Since the factor which causes the prolonged discharge is the absence of Ca ions, it is to be expected

that solutions of sodium oxalate or citrate would produce a much greater effect than NaCl alone. This is true for the stimulation of muscle fibres and Loeb [1905] has shown that application of an oxalate solution to the skin of a frog with intact spinal cord produces great reflex activity.

The first experiments were made with 0.6 p.c. Na oxalate solution. As this is hypotonic and contains no K it was replaced later by a solution made up of eight parts of Ca free Ringer and two parts of 1.2 p.c. Na oxalate, but as far as could be observed the results were the same.

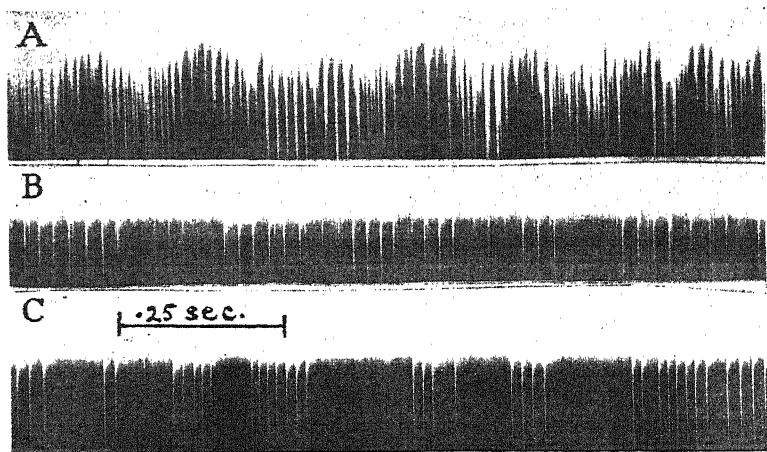


Fig. 3. Resting discharge (A) in 0.6 p.c. Na citrate (B and C) during recovery after isotonic oxalated solution. (B) 30 min., and (C) 60 min. in Ringer. All records at same speed.

The main result is a development of continuous activity in all the sense organs. The first noticeable effect is an increase in the response to a test stimulus (*e.g.* 5 g. wt. or light touch) and then a resting discharge develops (the skin being under slight tension). It appears first in nerve fibres giving small impulses conducted slowly, but soon the larger impulses appear and within 30–40 min. all the sensory endings in the skin seem to be discharging at a high frequency. The continuous activity lasts for about 5 or 6 hours and the frequency is apparently unchanged. Some of the records (Fig. 3 A) give the impression that the impulses in several fibres are discharged synchronously, as they may be in damaged nerve or muscle [Adrian, 1930; Adrian and Gelfan, 1933]. At the end of 5 or 6 hours the end organs seem to fail, one after the other,

and when all traces of the discharge have disappeared the skin is found to be inexcitable to any form of stimulation.

If Ringer is substituted for the oxalate solution before this failure has occurred the discharge subsides gradually but some of the end organs may still be discharging after 30 min. in Ringer (Fig. 3 B). Towards the end a single end organ may remain in action, but its activity is often intermittent (Fig. 3 C).

A few experiments have been made with the antidromic skin preparation in order to analyse the discharge in the individual nerve fibres. In this preparation it was found that the effect took longer to develop and lasted for about 1 hour only, unless the skin area giving the antidromic impulses was repeatedly irrigated with the oxalate solution. This may well be due to the skin having an intact blood supply.

With this preparation the frequency of the discharge in single nerve fibres was found to be as a rule between ten and forty per sec. It remains practically unchanged so long as the end organ is active. It is irregular below twenty to twenty-five per sec. but fairly regular above this. The frequency can be increased by touching or stretching the skin, and once a higher frequency is obtained it persists for a long time although the skin is allowed to relax completely.

The same results have been obtained with solutions of sodium citrate instead of oxalate.

#### DISCUSSION.

The action of the oxalate solution probably differs from that of the NaCl solution only in so far as it secures a more complete removal of Ca from the skin. Thus a sensory ending of the rapidly adapting type shows the following changes when the  $\text{Ca}^{++}$  ion concentration is progressively lowered. At first the discharge to a prolonged stimulus, instead of declining rapidly, persists for a greater and greater time. A stage in which the discharge coincides with the stimulus is soon followed by one in which there is a more and more prolonged after-discharge, and eventually a touch is enough to produce a discharge of high frequency lasting for 10 min. or more. At this stage the removal of the stimulus has little or no effect on the activity of the ending. As far as the stimulus is concerned the excitation is not now a reversible process, though the activity declines gradually and ceases sooner or later.

This stage recalls the action of NaCl solution on muscle fibres, for Adrian and Gelfan found that before spontaneous activity appears,

there is a period in which touching or stretching the fibres gives a discharge which may cease when the stimulus is removed but often outlasts it for a variable time. They pointed out that this behaviour suggested that of a sensory ending in which the mechanism of excitation had become brittle and inflexible instead of responding smoothly to the rise and fall of the stimulus.

Thus the three classes of excitable structure, muscle fibre, rapidly adapting and slowly adapting sense organ can be arranged in order with regard to mechanical stimulation and to the effects of diminished calcium. Normally a muscle fibre does not respond at all unless the stimulus is of damaging intensity; in NaCl it may give an abrupt, ungraded response which often outlasts the stimulus, and eventually prolonged discharges appear without any stimulation.

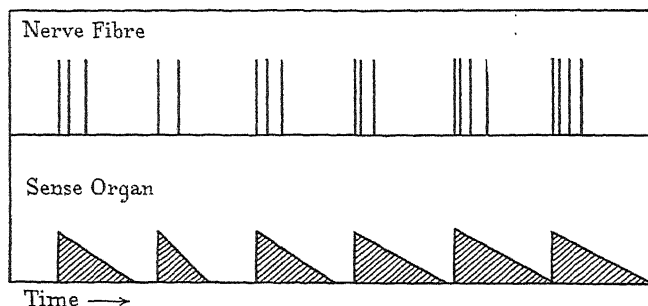


Fig. 4.

The rapidly adapting end organ responds normally by a brief discharge which declines at once although the stimulus is continued. In sodium chloride the adaptation becomes less and less rapid and ultimately the discharge, once it has been started by the stimulus, continues for a time which seems to depend only on the general state of the ending. At this stage there is not much difference between the response of the muscle fibre and of the nerve ending, but the latter preserves a little of its specialized properties, for its discharge can still be increased in frequency by an increase in the stimulus. A slowly adapting end organ such as a muscle spindle shows the most flexible discharge mechanism, for it combines a slow rate of adaptation with an excitatory process which follows every variation in the stimulus. It needs no reduction in Ca ions to enable it to give a prolonged discharge, and the discharge ceases at once when the stimulus is withdrawn.

The present records show one other point of interest, the development of the grouped discharge in the absence of Ca. The slow rate of adaptation seems to be associated with the tendency for each pulse of activity in the end organ to discharge not one but several impulses down the nerve fibre. The impulses are usually grouped as they would be if the pulse of activity which arouses them starts at its maximum intensity and declines gradually. Thus the lack of Ca prolongs the duration of the whole discharge and also prolongs the effect of each active period in the end organ. The relation between nerve ending and nerve fibre activity may be represented as in Fig. 4. The diagram recalls the slow potential waves found by Adrian and Gelfan in muscle fibre at the region of origin of a rhythmic discharge. In that case the waves rise slowly, but a series of potential waves rising steeply and declining slowly would account very well for the grouped discharges in the present case.

#### SUMMARY.

When the frog's skin is treated with solutions containing Na<sup>+</sup>, or Na<sup>+</sup> and K<sup>+</sup>, but no Ca<sup>++</sup>, there is a much less rapid adaptation of the sensory endings to a prolonged stimulus. Eventually the discharge, once started, may continue long after the stimulus has ceased. These changes develop sooner in solutions containing oxalate and the endings may remain in continuous activity for 5 or 6 hours. The impulses are often discharged in groups. The results are discussed in relation to the behaviour of muscle fibres and of sense organs of the slowly adapting type.

I am greatly indebted to Prof. E. D. Adrian for suggesting the work and for help in the preparation of this paper and to Mr B. H. C. Matthews for constant advice.

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## THE RESPIRATORY QUOTIENT OF THE EVISCERATE CAT.

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It is well known that the respiratory quotient of a spinal or decerebrate animal is higher after abdominal evisceration. This raised quotient has been observed in certain instances to attain a value of unity [Porges and Salomon, 1910; Best, Dale, Hoet and Marks, 1926] which has been accepted as added support for the view that the oxidative metabolism of skeletal muscle involves carbohydrate alone. On the other hand, respiratory quotients below unity have been observed for the abdominally eviscerated animal [Markowitz, 1928; Irving and Foster, 1930].

Evidence from other sources is also divided in regard to this question. The work of Meyerhof and his colleagues [1920, 1924] indicates that the quotient for the isolated muscle of the frog is unity, while Takane [1926], from the same laboratory, obtained a mean quotient of 0.79 for strips of rat's diaphragm.

Doisy and Beckmann [1922], Himwich and Castle [1927] and Himwich and Rose [1929], by analysis of arterial and venous blood of the limb muscles, obtained values for the respiratory quotient of mammalian muscle which are approximately those obtained with the intact animal.

It has frequently been suggested that the observed quotient is unreliable as a guide to the nature of the oxidative metabolism of a muscle preparation because of the variations in the  $\text{CO}_2$  output which may occur through changes other than those involving oxidation [Verzár, 1911; Murlin, Edelmann and Kramer, 1913; Krogh, 1916; Kilborn, 1928; Kilborn, Soskin and Thomas, 1928; Bornstein, 1929; Ferguson, Irving and Plewes, 1930].

There are two such changes, which are, however, not independent, namely a "blowing off" of  $\text{CO}_2$  by over-ventilation, and a displacement of  $\text{CO}_2$  from chemical combination in the tissues by accumulating lactic



acid. It is known that lactic acid will cause an increased ventilation, and that over-ventilation will in turn cause an increase in lactic-acid production [Eggleton and Evans, 1930]. The purpose of this paper is to discuss the relationship which has been observed to exist between the changes in lactic acid and  $\text{CO}_2$  concentration of the blood and the respiratory quotient.

#### METHODS.

The methods employed throughout were those described by Anderson, etc. [1931]. The respiratory metabolism of the cat, decerebrated under ether and abdominally eviscerated, was measured by the Douglas bag method. Blood lactic acid was estimated by the method of Friedmann, Cotonio and Shaffer, and the blood  $\text{CO}_2$  content of samples of blood, kept from contact with air, was determined with the van Slyke manometric apparatus.

#### RESULTS AND DISCUSSION.

The closeness of the relationship between the lactic-acid accumulation in the blood and the loss of  $\text{CO}_2$  therefrom was shown by Kilborn, Soskin and Thomas [1928], employing the spinal cat. In the present work this relationship has been investigated in some forty experiments, the results of which are typified in Fig. 1 (Exps. 34, 39). Following decerebration, the blood lactic acid may be high (Fig. 2, Exp. 142), but rapidly falls to a minimum value at which it may remain with only slight increase over a number of hours. On evisceration, however, there is an increase in blood lactic acid, which is paralleled by a decrease in  $\text{CO}_2$  and a rise in the respiratory quotient. This sudden increase is, however, of short duration and gives place to one which is more gradual or even to a slight or temporary decrease (Exps. 39, 42), the fall being accompanied by an equally small increase in the blood  $\text{CO}_2$ . When the lactic acid and  $\text{CO}_2$  of the blood have reached values of about 80 mg./100 c.c. and 25–30 c.c./100 c.c. respectively, the increase of lactic acid and decrease of  $\text{CO}_2$  are suddenly accelerated. This point of change has been termed the "break point."

In order to determine the relationship of these changes to the respiratory metabolism, the figures obtained in six experiments (31, 33, 34, 36, 39 and 41) have been subjected to a careful examination. In Fig. 3 are plotted the points representing all the simultaneous values for lactic acid and blood  $\text{CO}_2$  concentrations, and their degree of correlation,  $r$ , has been determined by the Karl Pearson formula. The

degree of negative correlation is high,  $r$  being  $-0.93 \pm 0.014$ . In Fig. 4 all these values have been grouped in sub-ranges of small magnitude (10 mg. of lactic acid and 5 c.c. of  $\text{CO}_2$  per 100 c.c. of blood) and the mean of each sub-range plotted. The line drawn through these points,

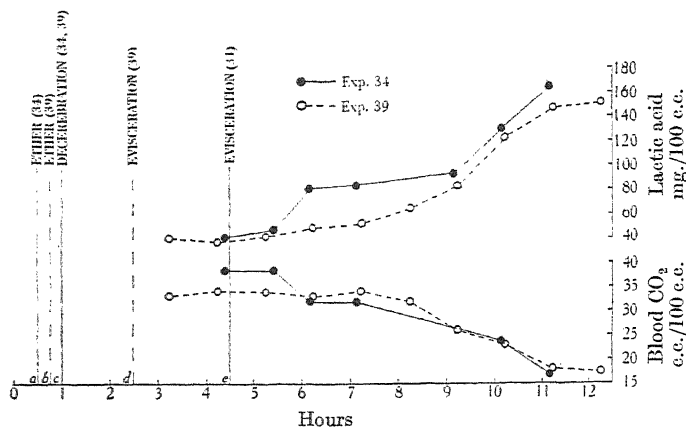


Fig. 1. The relationship of blood lactic acid to blood  $\text{CO}_2$  in the decerebrate-eviscerate cat.

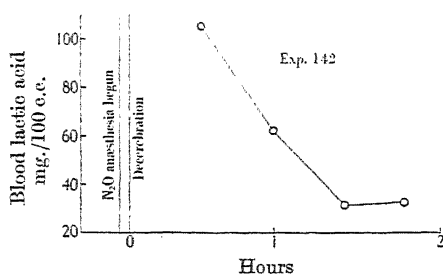
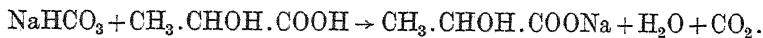


Fig. 2. Change in blood lactic acid concentration immediately following decerebration.

in Fig. 4, is not an attempt to apply the best-fitting straight line, but is an expression of the relationship between the quantities of lactic acid neutralized and the volume of  $\text{CO}_2$  displaced in the simple double decomposition reaction:



It is apparent that 1 g. molecule (90 g.) of lactic acid will displace 22.4 litres of  $\text{CO}_2$  at s.t.p., or 90 mg. will displace 22.4 c.c.

Whether or not the  $\text{CO}_2$  is displaced from the blood by this reaction, it is evident that the two changes, lactate increase and  $\text{CO}_2$  decrease, progress at rates which have a stoichiometric relationship, and it is to

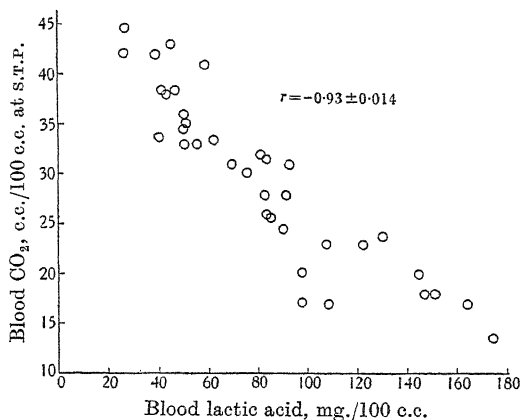


Fig. 3. The values of lactic acid and  $\text{CO}_2$  of the blood observed simultaneously at frequent intervals in six experiments.

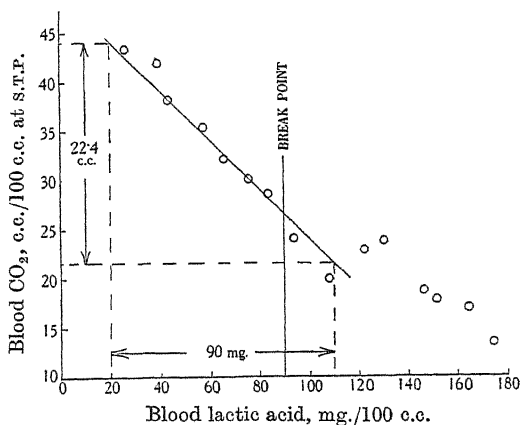
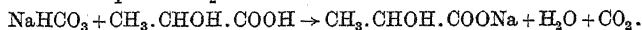


Fig. 4. The circles represent the concurrent changes in the concentrations of lactic acid and  $\text{CO}_2$  of the blood. The straight line represents the quantitative relationship between the lactate and displaced  $\text{CO}_2$  in the reaction



be expected that the observed respiratory quotient will be higher when the rate of fall of blood  $\text{CO}_2$  is greater; an examination of the observed respiratory quotient values for these six experiments shows this to be the case. Coincident with the decrease in blood  $\text{CO}_2$  on evisceration, is a

rise in the respiratory quotient which, during the subsequent period of slow accumulation of lactic acid, as in Exps. 33 and 39<sup>1</sup>, may maintain a fairly constant value, only to rise with the increase in rate of blood CO<sub>2</sub> loss after the "break point."

It is not a low blood CO<sub>2</sub> content which is associated with a high respiratory quotient, but a rapidly falling CO<sub>2</sub> concentration. In Fig. 5 are shown the observed respiratory quotient for these six experiments; each value, irrespective of the time in the history of the experiment during which it was observed, is plotted against the rate at which the blood CO<sub>2</sub> was decreasing at the time. The quotient is obviously high

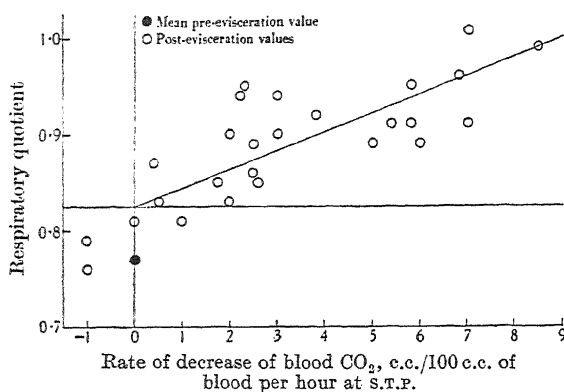


Fig. 5. The relationship between the R.Q. and the rate of decrease of blood CO<sub>2</sub> in the decerebrate-eviscerate cat. Two values found are not plotted: (1) CO<sub>2</sub>, 23.0; R.Q., 1.16; (2) CO<sub>2</sub>, 12.6; R.Q., 0.98.

when the rate of blood CO<sub>2</sub> decrease is rapid<sup>2</sup>, and a splined line approximating to the best-fitting straight line cuts the zero point on the blood CO<sub>2</sub> axis at a level which represents a respiratory quotient of about 0.825. Hence, if the rise in the respiratory quotient after evisceration is due to CO<sub>2</sub> displaced from the tissues by a non-oxidative process, the extent of which is measurable by the blood CO<sub>2</sub> loss, we can assume that the true oxidative quotient of the eviscerate preparation is somewhere

<sup>1</sup> Markowitz [1928] observed that the respiratory quotient of eviscerated dogs was lowered by the injection of glucose. This is in agreement with the finding that in the eviscerate cat the injection of fluid is attended with a delayed accumulation of lactic acid and a low respiratory quotient, probably to be explained as due to the maintenance of a high blood-pressure and a rapid circulation resulting from a lowered blood viscosity.

<sup>2</sup> It is of interest that Himwich and Castle [1927] observed only very slight changes in the CO<sub>2</sub> content of successive blood samples in their experiments in which they found the respiratory quotient of the resting skeletal muscle of the dog to be less than 0.8.

in the region of 0.8. Quotients of this order have been observed in the eviscerate preparation where the blood-pressure was well maintained (as in Exp. 39, in which the animal received an infusion of glucose solution) and the blood  $\text{CO}_2$  content was not suffering any significant diminution. Irving and Foster [1930] express their belief that the true respiratory quotient of the spinal eviscerate cat is approximately 0.8. The smallness of the number of values here examined makes it difficult to be precise as to this probable basal value. In Fig. 5, in black, is shown the respiratory quotient for the non-eviscerate preparation, and the one quotient value for the eviscerate preparation which fell below this non-eviscerate quotient is one obtained during a period when the preparation, Exp. 39, had a decreasing blood lactic acid and increasing blood  $\text{CO}_2$ .

TABLE I. Experiments 31, 33, 34, 36, 39, 41.

- a. Observed respiratory quotient.  
 b. Observed  $\text{CO}_2$  expired: c.c. at s.t.p. per kg. per hour.  
 c. Calculated  $\text{CO}_2$  output to give, with the observed  $\text{O}_2$  consumption, a respiratory quotient of 0.825: c.c. at s.t.p. per kg. per hour.  
 d. Volume of  $\text{CO}_2$  which has to be subtracted from the observed  $\text{CO}_2$  output to convert the observed R.Q. to 0.825: c.c. at s.t.p. per kg. per hour.  
 e. Rate of fall of blood  $\text{CO}_2$ : c.c. per 100 c.c. of blood per hour.

	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>
<i>Exp. 31.</i>	1.16	296	248	48	23.0
	1.07	291	218	73	7.0
	0.94	262	222	40	3.0
<i>Exp. 33.</i>	0.91	251	229	22	5.4
	0.83	229	228	1	0.5
	0.86	226	218	8	2.5
	0.92	268	240	28	3.8
	0.95	295	256	39	5.8
<i>Exp. 34.</i>	0.87	250	237	13	0.4
	0.85	216	205	11	2.6
	0.96	299	268	31	6.8
<i>Exp. 36.</i>	0.98	255	215	40	12.6
	0.89	236	219	17	2.5
	0.91	157	142	15	7.0
	0.85	163	157	6	1.75
<i>Exp. 39.</i>	0.76	208	220	-12	-1.0
	0.81	—	—	—	0.0
	0.81	252	256	-4	1.0
	0.79	218	228	-10	-1.0
	0.83	—	—	—	2.0
	0.89	237	235	2	6.0
	0.90	310	285	25	3.0
	0.89	389	369	20	5.0
<i>Exp. 41.</i>	0.90	209	192	17	2.0
	0.91	253	224	29	5.8
	0.94	218	191	27	2.2
	0.95	302	262	40	2.3
	0.99	430	358	72	8.5

concentration. If we accept this value of 0.825 as the approximately true respiratory quotient of the eviscerate cat, the extra volume of  $\text{CO}_2$  required to raise the respiratory quotient from this basal value to the observed value may be calculated in each instance and correlated with the rate of fall of blood  $\text{CO}_2$  (Table I).

A preliminary examination of these values suggested that their relationship could probably be represented by a straight line. The scattered nature of the results made it desirable that the decision on this point, if such were possible, should be made by a qualified statistician and, by the courtesy of Dr J. F. Tocher, who kindly submitted the data to a thorough investigation, I am able to give the following report.

"A preliminary study of the individual observations was made, the means and standard deviations of both series being determined. The two straight lines were then fitted by the method of least squares. It was found that for both straight lines one observation was exceptional and, on examination, this was found to be the first observation. It was decided, therefore, to omit this observation from the series. Two other observations, the second and last, also appeared somewhat exceptional, but were included in the further study.

"The raw data are not suitable for testing linearity owing to the small number of observations in both series. In order, therefore, to carry out the test satisfactorily, the two series were divided into sub-ranges. If  $x$  = rate of elimination of  $\text{CO}_2$  from blood in c.c. per 100 c.c. of blood per hour, all observations of  $x$  from -2 up to and including zero were taken and the mean found. Similarly, the mean value of  $x$  was found for the observations above zero up to and including 2 c.c., above 2 c.c. up to and including 4 c.c., and so on, the observations above 8 c.c. being taken together in the last group. Thus we have six sub-ranges or categories of  $x$  with definite ranges. The values of  $y$  = the volume of  $\text{CO}_2$  expired in excess of the basal rate, were classified sub-ranges; these, commencing with the lowest being below -15 up to and including -10, below -10 up to and including -5, etc. Thus the observations were classified in steps of 5 c.c., a grouping warranted owing to the scattered nature of the data. The data were now in a condition for statistical analysis and the observations were recorded in a table of double entry and the mean, standard deviation, correlation coefficient and correlation ratio determined. It was found that the correlation between the two series was fairly high. The mean of the  $y$  arrays of  $x$  were plotted and the straight line  $y_x = \bar{y} + \frac{ry}{x} (x - \bar{x})$  calculated. The observed mean of arrays and the best-fitting straight line are shown on the accompanying diagram (Fig. 6). The valid tests for goodness of fit were applied and it was found, for the small number of observations made, that the theoretical straight line was a reasonable fit to the data. In other words, when sufficiently broad sub-ranges of both series are taken, the volume of  $\text{CO}_2$  expired in excess of the basal rate is on the average proportional to the rate of elimination of  $\text{CO}_2$  from the blood."

When the probable experimental error is considered, it is apparent that the figures for  $\text{CO}_2$  in excess of the basal rate may vary within the limits of 5 c.c. per kg. per hour: the grouping together and averaging of the values within this range is, therefore, desirable and justifiable. Accepting the relationship as linear, it is to be observed, from Fig. 6, that the loss of an additional 1 c.c. of  $\text{CO}_2$  from 100 c.c. of arterial blood per hour is accompanied by the addition to the expired air of 4.5 c.c. of  $\text{CO}_2$  per kg. of animal weight per hour, over and above that necessary to maintain the respiratory quotient at 0.825. If we assume that the elevation of the respiratory quotient above this value is due to the

CO<sub>2</sub> displaced by lactic acid from the tissues and that the density of the blood is 1.06, these figures show that the equivalence of  $\frac{106 \times 4.5}{10} = 47.7$  p.c. of the body weight of the preparation, including the blood, is losing CO<sub>2</sub> at the same rate as the blood. If this is so, the figures obtained in these experiments offer a complete explanation of the variations in respiratory quotient after evisceration. The weight of the skeletal muscles of the cat according to the figures of Ferguson, Irving and Plewes [1929] amounts, on the average, to 44 p.c. of the total body weight. Higher figures are recorded by Best, Hoet and Marks [1926]. Allowing for the weight of viscera removed, the muscles therefore comprise about 49 p.c. of the weight of the eviscerate preparation, while the blood may be assessed at from 5 to 6 p.c. [Ferguson, Irving and Plewes, 1930]. On the assumption that muscle loses CO<sub>2</sub> at approximately the same rate as the blood, it is possible from these figures to explain the heightened values for the respiratory quotient, but it yet remains to be seen whether this assumption is justified.

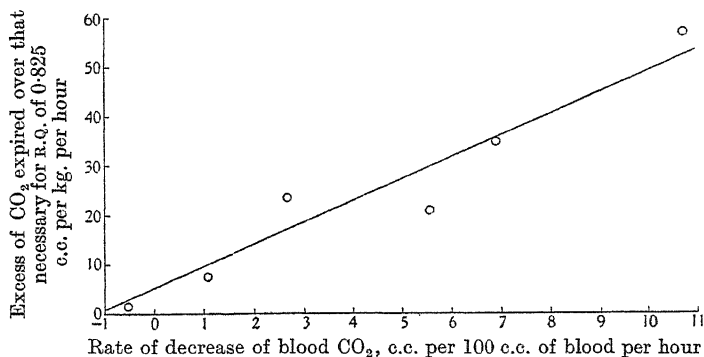


Fig. 6. The relationship of the rate of decrease of blood CO<sub>2</sub> to the excess of CO<sub>2</sub> expired over that necessary (with the observed oxygen retention) to maintain an R.Q. of 0.825.

It is of interest to speculate as to where the lactic acid comes from, and whether or not the CO<sub>2</sub> is lost as a result of the acid accumulation. The evidence so far obtained merely indicates that there is a concurrent equimolecular rate of change in the concentrations of CO<sub>2</sub> and lactate of the blood. The question as to whether either of the two processes, lactic acid increase or CO<sub>2</sub> decrease, can be considered as cause or effect remains open<sup>1</sup>. If we are to assume that more than 40 p.c. of the preparation is losing CO<sub>2</sub> at the same rate as the blood, as the result of a buffering process, the maintenance of a linear relationship between blood lactic

<sup>1</sup> The view that the lactic acid is produced as a result of hyperpnœa and is not its cause can be accepted for the decerebrate preparation only if some explanation of the cause of the hyperpnœa is forthcoming. The possibility of direct stimulation of the respiratory centre by a blood clot has been considered and the brain stems examined after each experiment without any results justifying this explanation. It has been stated that the activity of the respiratory centre is increased after decerebration [Winton and Bayliss, *Human Physiology*, 1930, p. 429].

acid increase and  $\text{CO}_2$  decrease must mean that the tissue which shares with the blood the rôle of buffering, must make with the latter a single system, the diffusion of lactic acid between them being rapid. This finding is further borne out by Fig. 5. If we accept the excess  $\text{CO}_2$  expired (over that necessary to maintain the respiratory quotient at 0.825) as an index of the part played by  $\text{NaHCO}_3$  as a buffer, it is apparent that with any variation in the proportionate part played by blood bicarbonate in this process, the relationship between the excess  $\text{CO}_2$  and the rate of decrease of blood  $\text{CO}_2$  will also vary. That is to say, if the blood plays a proportionately greater part in buffering after the "break point" than before it, values, in Fig. 5, representing conditions after the "break point," should lie below the straight line which represents conditions before it. Such is not the case.

To sum up, the evidence given supports the view that the raised respiratory quotient (above a value in the region of 0.825) observed in the eviscerate cat is due entirely to, and can be explained quantitatively by, the  $\text{CO}_2$  eliminated from the tissues of the preparation, a process which is accompanied by the accumulation of an equivalent quantity of lactate. The most obvious source of  $\text{CO}_2$  other than the blood, on the grounds of size and chemical nature, is skeletal muscle. Attempts, however, to obtain direct evidence of a loss of  $\text{CO}_2$  by muscle which would account for the extra  $\text{CO}_2$  expired beyond that coming from the blood have not given confirmatory results. In an attempt to find the source of the  $\text{CO}_2$  loss from the over-ventilated cat under amytal, Ferguson, Irving and Plewes [1929] measured the decrease in muscle and blood  $\text{CO}_2$ , but were unable to find a sufficient diminution. On the other hand, when the animal was ventilated with a gas mixture of high  $\text{CO}_2$  tension, it retained a quantity of  $\text{CO}_2$  greater than could be accounted for by the increase in the  $\text{CO}_2$  content of the muscles and blood. From this it would seem that, although the  $\text{CO}_2$  lost during over-ventilation is not traceable directly to a loss from the muscles and blood, the assumption may be made that it is produced by a process that is not necessarily oxidative [Ferguson, Irving and Plewes, 1930]. Irving and Foster [1930], by direct muscle analysis, found losses of  $\text{CO}_2$  which were, however, inadequate to account for the high respiratory quotient values observed.

The view that the respiratory quotient of a muscle preparation during its normal metabolism is less than unity, removes what was apparently a distinction between the nature of its metabolism and that of the heart. Starling and Evans [1914] observed that the average



respiratory quotient of the heart-lung preparation was about 0.85. Similar figures have been obtained by Clark and his colleagues [1932] for frog's heart.

From these results it is clear that in the use of the eviscerate mammalian preparation in the study of carbohydrate metabolism, observations of the quotient, in absence of information regarding the rate of loss of  $\text{CO}_2$  by direct displacement from the tissues of the body, cannot be relied on in the accurate computation of metabolic changes.

#### THE RESPIRATORY QUOTIENT OF THE ABDOMINAL VISCERA.

On the assumption that the removal of the abdominal viscera does not alter qualitatively the oxidative metabolism of the tissues that remain, the observed change in respiratory quotient, from 0.77 to 0.825, must be due to the removal of an oxidative process for which the respiratory quotient is low. This quotient can be readily calculated from the following data:

	Decerebrate cat	Decerebrate eviscerate cat
Oxygen consumption (c.c. per kg. (nett wt.) per hr.)	360.00	245.000
Respiratory quotient	0.77	0.825
Weight (kg.)	2.0	1.800

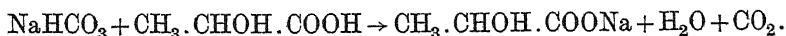
These values of oxygen consumption are in close agreement with those recorded by Evans, Tsai and Young [1931] for the decapitate cat, viz., 365 c.c. for the non-eviscerated and 250 c.c. for the eviscerated animal. From the figures given, and the assumption already made, it is obvious that the respiratory quotient of the removed viscera is

$$\frac{(360 \times 0.77 \times 2) - (245 \times 0.825 \times 1.8)}{(360 \times 2) - (245 \times 1.8)} = 0.69.$$

That the low value of this quotient is due to gluconeogenesis in the liver is the ready explanation which has already been discussed by other workers [Evans, Tsai and Young, 1931].

#### SUMMARY.

In the decerebrate-eviscerate cat, the quantitative relationship between the accumulation of lactate in the blood and the loss of blood  $\text{CO}_2$  is shown to be identical with that between lactate and displaced  $\text{CO}_2$  in the reaction:



A close relationship is demonstrated to exist between the rate of blood  $\text{CO}_2$  loss and the observed respiratory quotient, and evidence is adduced which supports the view that high quotients, obtained with the eviscerate preparation, are elevated above the true quotient by  $\text{CO}_2$  displaced from the tissues.

This problem was suggested by Prof. J. J. R. Macleod, whose constant help I gratefully acknowledge. I am indebted also to Dr R. A. Cleghorn and to Dr I. A. Anderson for much assistance in the experimental work.

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BY THE LATE N. U. MELDRUM AND F. J. W. ROUGHTON.

*(From the Physiological and Biochemical Laboratories, Cambridge.)*

*(Received August 2, 1933.)*

### INTRODUCTION.

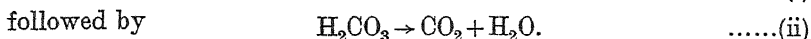
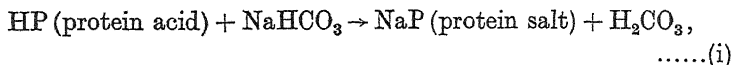
THERE have been two main theories as to the mode of carbon dioxide transport in blood: (*a*) that carbon dioxide is carried from the organs of the body to the lung mainly in the form of bicarbonate, and that when the blood reaches the lung the proteins of the blood acting as weak acids (expressible by the formula HP) convert the bicarbonate into carbonic acid ( $\text{H}_2\text{CO}_3$ ) which in turn dehydrates to water and dissolved carbon dioxide. The latter then volatilizes as  $\text{CO}_2$  gas into the air spaces in the lung from which it is eliminated by expiration; (*b*) that in addition part, and possibly the more important part physiologically, of the carbon dioxide is carried in direct reversible combination with the blood proteins. Of these theories (*a*) has been called the bicarbonate theory and has had the support of Zuntz (quoted by Haldane, 1922), Parsons [1919, 1920], Poulton [1920], L. J. Henderson [1928], Peters and van Slyke [1931], Stadie [1928, 1931] and their co-workers. The direct combination theory (*b*) has, on the other hand, been sponsored by Bohr [1909], Buckmaster [1917 *a*, *b*], Bayliss [1924], Mellanby and Thomas [1920], and by later workers to whom reference will be made below.

From 1917 to 1921 the problem was worked at intensively by numerous British physiologists, with the result that at the end of this period the bicarbonate theory, in the view of most writers, had established a decided lead over its rival. Thereupon the scene shifted to America, and there followed an exhaustive study of the acid-base equilibria in blood by van Slyke and various colleagues. These workers gave full data as to the titration curves of oxyhæmoglobin and reduced hæmoglobin, which, together with their data upon the  $\text{CO}_2$  dissociation curves, seemed to show that under physiological conditions the amount of  $\text{CO}_2$

in blood in forms other than  $\text{CO}_2$ ,  $\text{H}_2\text{CO}_3$  or  $\text{HCO}_3^-$  is negligible. It will, however, be noted below that both in this work, and in that of Stadie and his collaborators [1928, 1931], there are certain features which can only be explained by the simple bicarbonate theory, with the aid of rather forced assumptions. These apparent anomalies readily fall out if a certain proportion of carbon dioxide exists in some form other than  $\text{CO}_2$ ,  $\text{H}_2\text{CO}_3$ , or  $\text{HCO}_3^-$ , *e.g.* in direct combination with the blood proteins.

Up till 1925, attention had been given solely to the equilibrium states of the process, but in that year Hartridge and Roughton, quoted by L. J. Henderson [1925], following on their measurements of the velocity of oxygen combination and dissociation from hæmoglobin, pointed out the desirability of studying the kinetics of the carbon dioxide processes in blood. In 1926 this aspect was taken up by Henriques with a startling outcome.

According to the bicarbonate theory the detailed chemical reactions which lead to  $\text{CO}_2$  evolution in the lungs are as follows:



Of these reactions (i), being purely ionic, is very rapid, but (ii) is known from the work of Thiel *et al.* [1913 *a, b*], Faurholt [1924], and the recent paper of Brinkman, Margaria and Roughton [1933] to be rather slow. On the bicarbonate theory reaction (ii) must therefore limit the rate of  $\text{CO}_2$  escape in the expired air. Using the velocity constants of previous authors, Henriques (and others independently) calculated that the rate at which  $\text{CO}_2$  could be formed by reaction (ii) under physiological conditions would be far less than that actually observed in the expired air. Hence he concluded that either one or both of the following conditions must obtain: (*a*) there must be a catalyst for the reaction  $\text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O}$  in the blood; (*b*) the physiological transport of carbon dioxide in the blood must take place by some mechanism other than the bicarbonate one.

The most important of Henriques' [1928] experiments were those in which he compared the rate at which  $\text{CO}_2$  is evolved when hæmoglobin solutions, on the one hand, and serum solutions, on the other hand, were violently shaken *in vacuo*. He noted for the first time that  $\text{CO}_2$  came off much more quickly from the hæmoglobin solutions especially in the early stages of the shaking, whereas the rate of escape from serum was of the same order as that calculated from Faurholt's velocity constants for

the reaction  $\text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O}$ . He concluded that his results could not be explained by a catalyst mechanism, but required instead the existence of a very rapid reversible reaction between  $\text{CO}_2$  and hæmoglobin direct, analogous to the reaction between oxygen and hæmoglobin. To this hypothetic complex, which he suggested might be of a carbamate type, he gave the name carbhæmoglobin. Hawkins and van Slyke [1930], however, in experiments of a similar type found distinct evidence of a catalyst, and this was clinched by Brinkman and Margaria [1931], who showed that blood or hæmoglobin, even when diluted to 1 part in 20,000, accelerated markedly the evolution of  $\text{CO}_2$  from mixtures of bicarbonate with buffers of physiological  $p\text{H}$ . Finally in 1932 the present writers isolated from ox blood a white substance of which 1 part in 10,000,000 is active in accelerating the reaction. This solid extract, which is quite free from hæmoglobin and apparently also from hæmatin compounds, has the typical properties of an enzyme, and since it can be shown to be distinct from the other enzymes known to be present in blood, it has been given a special name: CARBONIC ANHYDRASE. Preliminary reports on its biochemical properties have already been given by us elsewhere [Meldrum and Roughton, 1932 *a, b, c*], and a more complete account of its preparation and properties is given below.

With such large amounts of carbonic anhydrase in the red blood corpuscles it no longer seems difficult to account for the rate of  $\text{CO}_2$  evolution in the expired air on the simple bicarbonate theory. This does not, however, prove that the bicarbonate mechanism is the sole mode of physiological transport; a definite conclusion as to the rôle of the hypothetical  $\text{CO}_2$  protein compounds is indeed needed, not only for the sake of its own interest and for possible importance, but also to enable us to assess precisely the rôle of carbonic anhydrase in blood. For reasons to be given later, we do not regard either the recent experiments of Henriques [1928, 1931 *a, b*, 1933] or of Margaria [1931] as decisive or complete, and we have therefore devised and carried out a new set of experiments, which seem to us less equivocal in their indications. Our results not only point to appreciable amounts of direct  $\text{CO}_2$  protein compounds especially at low temperatures (the amount diminishing very much as the temperature and/or the acidity is increased), but also suggest strongly that these compounds are of a carbamate type, as has indeed been hinted at by previous workers. The whole question is dealt with in a separate paper by us [Meldrum and Roughton, 1933 *b*] in the present *Journal*.

## SCOPE OF THE PRESENT PAPER.

We shall start by describing the method of measuring the catalytic activity, and pass thence to the various methods of separating the enzyme from hæmoglobin and the means adopted for its further purification. After summarizing the evidence that carbonic anhydrase is distinct, not only from hæmoglobin, but also from some other enzymes present in the blood corpuscles, we then proceed to some of the biochemical properties of the enzyme. Here we deal, in a preliminary way, with the nature of the enzyme and some of the factors which influence its stability and activity. We have made a fairly detailed study of the effect of various poisons and inhibitors, including metallic salts from most groups in the periodic table. In the last pages we give some further notes about the distribution of the enzyme, especially in foetal blood as compared with maternal blood; we also compare the carbonic anhydrase activity of unlaked corpuscles with that of laked corpuscles from the same blood. This last matter is obviously of great physiological importance as regards the action of the enzyme in circulating blood: our preliminary experiments show that there is need for much further study on these lines.

SECTION I. THE METHOD OF ESTIMATING THE CATALYTIC  
ACTIVITY OF THE VARIOUS PREPARATIONS.*The technique.*

A simple means of measuring the amount of enzyme present in the various media and preparations is clearly an indispensable preliminary to a study of the isolation and properties of the enzyme. The method adopted was to measure the catalytic effect upon the rate of evolution of  $\text{CO}_2$  from sodium-bicarbonate solutions when mixed with phosphate buffer of  $\text{pH}$  6.8, with the aid of the boat apparatus of Brinkman, Margaria and Roughton [1933] as used by them in their work on the kinetics of the reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$  in the absence of catalyst.

In one compartment of the glass boat-shaped trough (Fig. 1) are placed 2 c.c. of phosphate solution made by mixing equal volumes of  $M/5$   $\text{Na}_2\text{HPO}_4$  and  $M/5$   $\text{KH}_2\text{PO}_4$ , whilst in the other compartment are placed 2 c.c. of  $M/5$   $\text{NaHCO}_3$  dissolved in  $M/50$   $\text{NaOH}$ . The boat is attached by a well-fitting stopper and length of pressure tubing to a manometer, open at the other end to the air. Most of the experiments in this paper



were thus done at atmospheric pressure, instead of at reduced pressure as in the earlier paper.

After allowing time for temperature equilibration the boat is then shaken rapidly in a constant temperature water bath (usual temperature about  $15^{\circ}\text{C}$ .) and the evolution of  $\text{CO}_2$  followed by noting the reading of the manometer at 0, 5, 10, 15, 30, 45, 60, 90 and 120 sec. and thereafter at minute intervals if necessary. Provided that the first half of

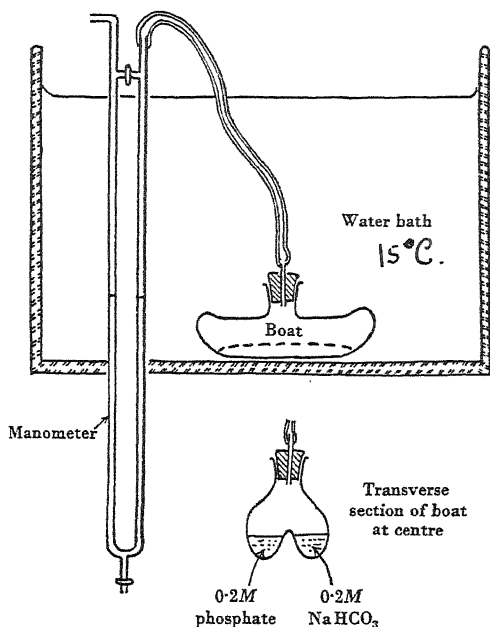


Fig. 1. Boat technique for measurement of enzyme activity.

the total  $\text{CO}_2$  is evolved in not less than about 15 sec., diffusion from liquid to gas phase does not prove to be a limiting factor, and the rate of  $\text{CO}_2$  evolution as observed is a true measure of the velocity of the chemical reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$ . A typical set of curves obtained with various amounts of goat's blood added to the phosphate-bicarbonate mixture is shown in Fig. 2 a.

#### *Calculation of the activity.*

In Fig. 2 b is plotted the average rate of  $\text{CO}_2$  evolution during the second quarter of the process (as given by the reciprocal of the time

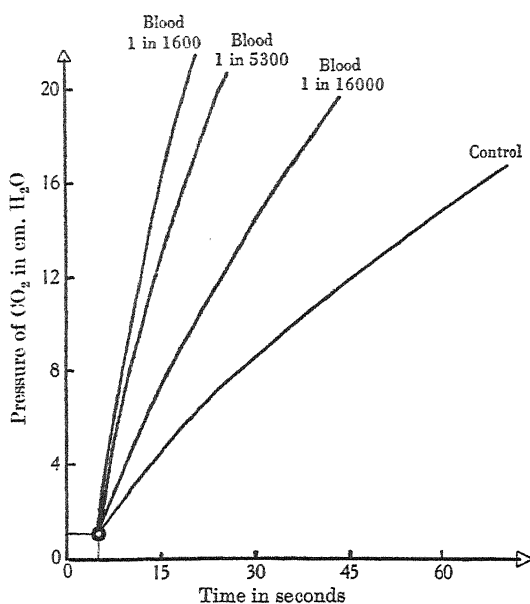


Fig. 2a. Effect of various amounts of goat's blood on rate of CO<sub>2</sub> output from phosphate-bicarbonate mixture.

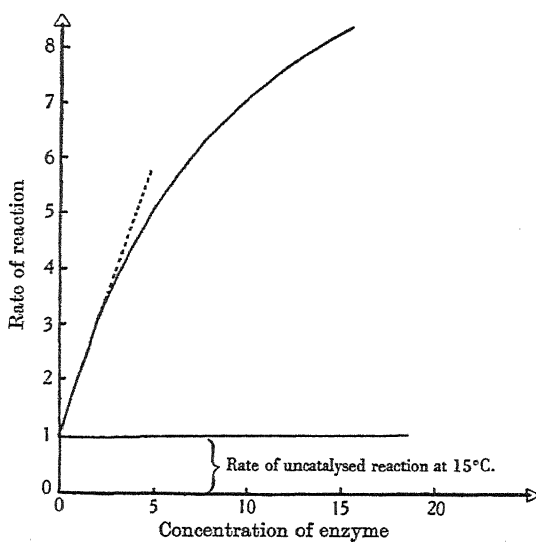


Fig. 2b. Relation between concentration of enzyme and effect on rate of reaction. [The rate of the reaction in absence of catalyst being taken as unity.]

taken) against the concentration of catalytic material added, the samples of the latter ranging from crude blood to relatively pure material.

Let  $R_0$  be the rate in absence of catalyst,  $R$  be the rate in presence of catalyst. Then it is clear from the curve that the fraction  $(R - R_0)/R_0$  is a suitable measure of the catalytic activity, for:

(a) It is proportional to the amount of catalyst added, to within  $\pm 5$  p.c., provided that the rate of the process is not too fast, *i.e.* the time is not less than 10 sec.

The breakdown of the linear relation at faster rates is certainly in part due to diffusion becoming a limiting factor in addition to chemical reaction velocity.

(b) It is independent of the nature of the added catalyst, *e.g.* whether it contains hæmoglobin or not.

(c) It is independent of the dimensions of the apparatus, which need not even be known.

(d) It eliminates initial errors due to the time taken for the bicarbonate and phosphate to mix after the shaker starts.

(e) The range of the pH change during the period is small and constant.

In ordinary cases it makes no difference whether the catalytic material is added to the phosphate or to the bicarbonate, or if it is distributed between both solutions.

It seems premature to define the unit of carbonic anhydrase activity until a full study of the kinetics of the enzyme reactions has been made. For the present it is proposed to assign the value  $E$  to that amount of enzyme which, when dissolved in 4 c.c. of the phosphate bicarbonate mixture used above, gives a value of  $(R - R_0)/R_0$  equal to 1 at 15° C. If the temperature differs slightly from 15° C., a correction can be made by applying a temperature coefficient (*i.e.*  $Q_{10}$ ) of 3.6 per 10° C. for the uncatalysed reaction and a  $Q_{10}$  of 2.1 for the catalysed reaction. This temporary unit  $E$  has the convenience that ox blood (the main type of blood used in our work so far) contains on the average about 1  $E$  per c.mm.

Whenever a test was made of the catalytic activity of any solution, a control experiment with phosphate and bicarbonate solutions alone was done as well. To get the most accurate result the amount of catalytic material added should be such as to increase the rate of the process about three to four times, since the relation between enzyme concentration and rate of reaction is still linear up to this point. In some tests, unfortunately a much larger amount of catalyst was used; in view of the shape

of the curve in Fig. 2*b* the results were correspondingly less accurate, even though the necessary corrections were applied.

The percentage yield in any given preparation from blood was calculated from a comparison of the activity of the preparation with that of the original blood diluted with an amount of water equal to the amount of aqueous fluid added to the blood in the course of the preparation.

## SECTION II. SEPARATION AND PURIFICATION OF THE ENZYME.

### *Separation from hæmoglobin.*

The finding of Brinkman, Margaria, Meldrum and Roughton [1932] that not all hæmoglobins are catalytically active, but that native globin prepared from an active hæmoglobin is fully active, suggested strongly that the active principle is not hæmoglobin itself, but some substance or some system which frequently, but not invariably, accompanies the hæmoglobin. That the latter is the case was settled very quickly by applying the classical methods of removing hæmoglobin with but little modification. Three methods, *A*, *B*, *C*, were tried, and all were successful. Of these the crude product of method *C* was used for nearly all the further work in this paper.

*Method A.* Mammalian erythrocytes (previously washed three times with isotonic saline) are lysed with water and diluted till the hæmoglobin is about 1 p.c. The aqueous solution is then saturated with chloroform and allowed to stand at room temperature in presence of excess  $\text{CHCl}_3$  with intermittent shaking till all the hæmoglobin is denatured. The usual time required is about 24 hours: a temperature of  $37^\circ \text{C}$ . hastens denaturation but destroys the enzyme. The protein and excess chloroform are centrifuged down and the enzyme solution poured off. It usually contains of the order of 50 p.c. of the carbonic anhydrase originally present, much catalase, and traces of hæmoglobin ( $> 1$  in 5000). This method is similar to that of Tsuchihashi [1923] for preparing catalase.

*Method B.* By following the method of Warburg and Christian [1931] for preparing the co-enzyme for the system which oxidizes glucose- $\delta$ -phosphate, carbonic anhydrase can readily be prepared. 200 c.c. washed horse erythrocytes are lysed at  $0^\circ \text{C}$ . with an equal volume of water and 350 c.c. of ice-cold alcohol added. Then 15 c.c.  $\text{CHCl}_3$  are added to the homogeneous mixture and the whole shaken for a few minutes at  $0^\circ \text{C}$ . The suspension is centrifuged and the centrifugate poured into 450 c.c. alcohol and 1200 c.c. ether, when a whitish precipitate gradually falls.

The supernatant liquid is sucked off, the solid collected on a filter and washed with absolute alcohol.

This enzyme preparation is very stable, it does not readily dissolve in water, but gives a highly active and fairly clear solution when 10 mg. are ground in a mortar with 1 c.c. of  $M/5$   $\text{Na}_2\text{HPO}_4$ .

The yield is only about 7 p.c., and the activity per mg. dry weight is about 350 *E* as measured by the method of Section II, assuming that the whole of the enzyme is in solution. The latter assumption is, however, probably unfair.

*Method C.* To 10 c.c. washed ox corpuscles in a centrifuge tube are added 6 c.c.  $\text{H}_2\text{O}$  and 4 c.c. ethyl alcohol. The mixture is then shaken at room temperature for about a minute with 5 c.c.  $\text{CHCl}_3$ . On centrifuging for about 10 min. at 3500 r.p.m. a three-phasic system is formed, consisting of a top layer of enzyme solution, a central layer of denatured protein, and a bottom layer of chloroform.

The enzyme solution contains traces of hæmoglobin and much catalase. The yield is on the average about 50 p.c. of the maximum. In different cases there were considerable variations from this figure both in upward and downward directions. The cause of this is not known but may be in part explained by varying amounts of enzyme adhering to the hæmoglobin coagulum. Rubbing up the latter with water gave a solution of high activity on the one occasion when the test was made. On evaporating the enzyme solution to dryness in a vacuum desiccator a brown solid enzyme preparation is obtained containing methæmoglobin and hæmochromogen compounds. The yield is about 1 g. from 100 c.c. corpuscles.

The enzyme is extremely stable in this form and can be kept for many months even if exposed to the air. The brown solid readily dissolves in water or salt solutions to yield a clear solution, having the properties of the original (unevaporated) solution. In aqueous solution the enzyme keeps for many weeks in the ice chest. The rate of destruction varies with different samples, but in general the enzyme loses  $> 20$  p.c. activity in 5–6 weeks.

The solution of enzyme thus prepared is known in the succeeding parts of this paper as "crude chloroform" preparation, and is used as the basis for further purification.

Some two dozen preparations were made by this method, which was found to work for the blood of other mammals besides ox. In one case with horse blood an 80 p.c. yield was obtained. In the earlier cases of the series a lower proportion of alcohol was used, but this led to much

slower precipitation of the hæmoglobin, several shakings with  $\text{CHCl}_3$  often being necessary before a colourless (*i.e.* hæmoglobin-free) supernatant fluid was obtained. The yield, however, was as great as when the proportion of alcohol mentioned above was used, the great advantage of the latter lying in the quickness of the method. The difference in speed of coagulation of the hæmoglobin forms, indeed, a striking demonstration: for the quickest results the proportion of 6 parts water to 4 parts alcohol should be adhered to closely.

#### *Purification.*

The further purification of the enzyme is best carried out starting from the crude chloroform preparation. Such a preparation contains two types of impurity: (*a*) dialysable; (*b*) non-dialysable.

The dialysable impurities are readily removable by ultra-filtration. For this purpose Bechhold's 6 p.c. and  $4\frac{1}{2}$  p.c. ultra-filtration membranes were purchased from Carl Schleicher and Schüll, and ultra-filtration by vacuum suction was found quite satisfactory, provided the fluid on the membrane is gently stirred.

The non-dialysable impurities: Attempts were first made to precipitate either the enzyme or the accompanying protein material with ammonium sulphate, but these were unsuccessful. In the case of crude hæmoglobin solutions most of the enzyme is carried down with the precipitated proteins, while in the case of the crude chloroform preparations a large and variable amount of enzyme is removed, probably by adsorption on precipitated material.

A series of adsorbents was next tried. Kaolin, permutit, four different sorts of charcoal, silk, cellulose and chalk were unsatisfactory. Very good results, however, were obtained with Willstätter's C- $\gamma$  alumina cream, and with a suspension of  $\text{Ca}_3(\text{PO}_4)_2$  (roughly 1 p.c. prepared by precipitation in the customary manner), filtering after each treatment with adsorbent. These adsorbents are both effective in removing selectively the other non-dialysable impurities, especially the traces of colouring (hæmatin) matter, without at the same time taking up serious amounts of carbonic anhydrase. The extent of the treatment must be adjusted for each sample.

The following is a protocol by N. U. M. of a purification by the  $\text{Ca}_3(\text{PO}_4)_2$  method:

100 c.c. ox corpuscles were washed three times with isotonic saline and then treated with 80 c.c. water, 20 c.c. alcohol and 150 c.c.  $\text{CHCl}_3$ . The mixture was shaken for some minutes, stood overnight at  $2^\circ \text{C}$ .,

centrifuged, shaken and centrifuged again. The supernatant fluid was filtered, volume of filtrate  $A = 108$  c.c. 25 c.c.  $A$  were treated with 20 c.c. of  $\text{Ca}_3(\text{PO}_4)_2$  suspension, and the mixture filtered. Volume of filtrate  $B = 40$  c.c.

30 c.c. of filtrate  $B$  were ultra-filtered on a 6 p.c. Bechhold membrane, being washed three times with about 13 c.c. water.

The residue on the membrane was made up to 40 c.c. with water = solution  $C$ .

The volume of the ultra-filtrate was 67 c.c. = solution  $C'$ .

0.05 c.c. of solution  $C$  was tested and found to have an activity = 6  $E$ .

5 c.c. of solution  $C$  evaporated to dryness at  $130^\circ \text{C}$ . gave a dry weight of 1.2 mg. Therefore the activity per mg. of the solid in solution  $C = 500 E$ .

N. U. M.'s purest preparation was made by  $\text{Al}(\text{OH})_3$  adsorption, the steps of the process being otherwise almost the same as in the previous example. In this case details are available as to the activity per mg. dry weight at three different stages of the preparation.

Activity of original ox corpuscles per mg. dry weight = 4.6  $E$   
(assuming water content of corpuscles = 60 p.c. by weight).

Crude chloroform preparation shows 48 mg. dry weight per 5 c.c.

Activity per mg. dry weight = 90  $E$ .

The dialysable impurities were removed by ultra-filtration and the final solution after three treatments with an equal volume of  $\text{Al}(\text{OH})_3$  cream had an activity per mg. dry weight = 1730  $E$ . 5 c.c. of this final solution evaporated at  $100\text{--}150^\circ \text{C}$ . contained 1 mg. dry weight, and was thus almost the same in concentration as the final solution of the previous case, although the activity was about 3.5 times greater.

Comparison of the activity per mg. dry weight of the final preparation with that of the original corpuscles shows that a concentration of about 400-fold was attained.

The figures given show that 1 part of the pure solid (by weight) in 7,000,000 parts of solution is sufficient to double the rate of  $\text{CO}_2$  evolution, and that during the first 15 sec. 1 g. of enzyme is responsible for the production of 825 g. of  $\text{CO}_2$ . This means a rate of 1.24 mols  $\text{CO}_2$  per sec. per g. of enzyme.

If the enzyme is pure, and is a protein (*vide* Section III) of the minimum molecular weight found by Svedberg, viz. 34,000, then 1 mol enzyme would catalyse the formation of  $4 \times 10^4$  mols  $\text{CO}_2/\text{sec}$ .

*Further purification of the enzyme.*

The fact that our best preparation has the same order of activity as the most active enzymes of other workers makes it perhaps unlikely that much further purification will be possible. We have not yet, however, used in a systematic way one of the classical weapons of enzyme purification, namely adsorption of the enzyme, followed by elutriation of the enzyme from the adsorbent. Preliminary work suggests that the enzyme is to some extent adsorbable by alumina C- $\gamma$  cream, especially if the impurities adsorbed by the latter are first removed. Thus in two cases we were able by appropriate treatment to adsorb as much as 40 p.c. of the enzyme on alumina. We also found on one occasion that from the purest solution the enzyme was partly adsorbed by kaolin, and could be in part elutriated therefrom by means of  $M/15 \text{ Na}_2\text{HPO}_4$ .

Fractional ultra-filtration is another possible line. Thus in one experiment the crude chloroform preparation was shaken with alumina, filtered and the filtrate then ultra-filtered through a 6 p.c. Bechhold membrane. The solution which came through the ultra-filter had an appreciable activity. In the two other cases, the crude chloroform preparation was ultra-filtered directly through a  $4\frac{1}{2}$  p.c. Bechhold membrane. The solution which came through had respectively 13 and 25 p.c. of the activity of the crude chloroform preparation in the two cases.

SECTION III. THE "INDIVIDUALITY" AND SOME PROPERTIES  
OF THE ENZYME.

*The evidence that carbonic anhydrase is distinct from hæmoglobin.*

Earlier workers on the  $\text{CO}_2$  catalyst in blood inclined to the view that it was identical with hæmoglobin. Against this there are now three strong arguments:

(a) There is no correlation between the distribution of hæmoglobin and of carbonic anhydrase in lower organisms. Examples of this lack of correlation have already been given by Brinkman, Margaria, Meldrum and Roughton [1932], and further instances occur in Brinkman's [1933] paper in the present number of this *Journal*. A striking case of the same kind will be found in a later section of this paper, where it is shown that in foetal blood of goats the ratio carbonic anhydrase content/hæmoglobin concentration, *i.e.*  $E/\text{Hb}$ , may be only 1-2 p.c. of the value in the mother blood of the same animal.



(b) It is now possible by the simple methods of Section II to prepare highly active preparations of the enzyme, which are completely free from hæmoglobin.

(c) We have been able in the case of two species of blood of which the hæmoglobin crystallizes easily—namely, horse and rat—to effect a considerable separation of hæmoglobin from the enzyme merely by crystallization.

Thus in the case of horse hæmoglobin, a single crystallization gave a value of  $E/Hb$  about three times greater in the mother liquor than in the crystals. In the case of the rat sample the first mother liquor, after crystallization at  $0^{\circ}C.$ , had only one-seventh the hæmoglobin concentration of the original blood, but yet had nearly the same concentration of carbonic anhydrase. Two further crystallizations gave a preparation of hæmoglobin with an  $E/Hb$  value only 7 p.c. of the  $E/Hb$  in the original blood. It seems likely therefore that by very many recrystallizations hæmoglobin might be obtained free from carbonic anhydrase as well as from catalase, as has already been done as regards the latter only.

The properties of such hæmoglobin, if not too largely contaminated with “inactive material,” would be interesting to compare with those of hæmoglobin “purified” by the usual means.

#### *Freedom from hæmatin compounds.*

No trace of hæmatin compounds could be detected in the purified alumina preparations, even when examined in very thick layers by spectroscopic methods.

#### *Distinction of carbonic anhydrase from other enzymes of the blood, etc.*

(a) *Catalase.* There is no correlation between the distribution of catalase and carbonic anhydrase: thus, for example, there is much catalase in yeast, but usually no detectable carbonic anhydrase. Furthermore we found that our crude chloroform preparations, which contained abundant catalase as well as carbonic anhydrase, were freed entirely of catalase by means of the  $Al(OH)_3$  adsorption technique. Thus the filtrate obtained after the usual alumina treatment still contained a high proportion of carbonic anhydrase, but no catalase.

(b) *Peroxidase.* Both the guaiacum and benzidine tests are negative, when applied to our pure preparation.

(c) *Oxidase.* Preliminary observations failed to detect blueing of tetramethylparaphenylenediamine, nor was any catalysis of oxidation of reduced cytochrome *c* to be observed.

So far then we feel justified in believing that carbonic anhydrase is a new enzyme, distinct from any previously described enzyme or system.

*Protein tests, etc.*

The simple characteristics of the solid obtained by evaporating the crude chloroform preparation to dryness have already been described. The active solid obtained by evaporating the purest solutions to dryness at low temperatures is colourless and almost transparent.

The presence of proteins in the various preparations was looked for by the usual tests. The results are shown in Table I, which also includes the  $\alpha$ -naphthol test.

TABLE I.

*A* = Crude chloroform preparation.

*B* = Solution of solid prepared as in Section II, method *B*.

*C* = Purest solution ( $\text{Al}(\text{OH})_3$  filtration treatment, etc.).

Test	Result		
	<i>A</i>	<i>B</i>	<i>C</i>
Millon's	++	++	++
Xantho-proteic	++	++	++
Sulphur	++	++	?
Glyoxylic	+	+	+
Biuret	.	.	+
$\alpha$ -Naphthol	+	+	+

++ = Marked reaction.

+ = Moderate to slight reaction.

? = Doubtful.

Since in relatively pure solution the enzyme is only partially and variably precipitated by saturation with ammonium sulphate, and tends furthermore to pass through ultra-filtration membranes to an appreciable extent, it is probable that the enzyme, if a protein, is a rather small molecule.

Further work on such matters as the chemical composition of the enzyme, etc., its molecular weight, solubility and acid and base-binding properties, has been postponed until the efforts at maximum purification shall have been exhausted.

#### SECTION IV. THE STABILITY, SCOPE OF ACTION, AND KINETICS OF THE ENZYME.

##### *Stability.*

(a) *To temperature.* The stability of the enzyme, in three different grades of purity, was tested by exposing the solutions to various tempera-

tures for 30 min., and then measuring the activity as in Section I. The result was as follows:

	20° C.	55° C.	65° C.
Lysed corpuscles (1 part in 80 parts water)	No loss	30 p.c. loss	Completely destroyed
Crude $\text{CHCl}_3$ preparation	"	—	"
Very pure $\text{Al}(\text{OH})_3$ preparation (in water)	"	No loss	60 p.c. loss

(b) *To pH.* The same three preparations were taken to various *pH*'s for 30 min. periods, and then tested for activity.

	Citrate phosphate buffer			Borate buffer <i>pH</i> 11	<i>N</i> /100 NaOH <i>pH</i> 12	<i>N</i> /10 NaOH <i>pH</i> 13
	<i>pH</i> 3	<i>pH</i> 4	<i>pH</i> 6-10			
Lysed corpuscles	Completely destroyed	No loss	No loss	—	—	Completely destroyed
Crude $\text{CHCl}_3$ preparation	"	"	"	No loss	No loss	"
$\text{Al}(\text{OH})_3$ preparation	"	"	"	—	30 p.c. loss	"

*The scope of the reactions catalysed by carbonic anhydrase.*

The reactions  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$  and  $\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^-$ . These reactions, *i.e.* the reverse to that so far dealt with in this paper, are of course also catalysed by the enzyme. The boat technique was again used, but in this case a two-compartment vessel was not necessary. 4 c.c. of a suitable buffer were placed in the boat, the latter connected by one lead through its cork to the manometer, and by a second lead through a very fine capillary and a rubber tube with pinchcock to a vessel containing  $\text{CO}_2$  gas at about  $1\frac{1}{2}$  atmospheres. About 25 sec. before shaking was due to begin the pinchcock was opened and  $\text{CO}_2$  allowed to stream into the boat until a suitable pressure, as shown by the manometer reading, was reached. This usually took 15 sec.

After 10 sec. more, shaking was started and the uptake of  $\text{CO}_2$  recorded by the manometer readings at 0, 5, 10 and 15 sec., etc.

For higher  $\text{CO}_2$  pressures than 50 mm. Hg, the water in the manometer was replaced by mercury and the technique slightly altered.

Fig. 3a shows the catalytic effect of 1 part crude  $\text{CHCl}_3$  enzyme in 40,000 parts solution upon the rate of uptake of  $\text{CO}_2$  by 0.2 *M* phosphate buffer *pH* 7.6 at 17° C. The reaction is accelerated about eight-fold. This may be compared with the fourteen-fold acceleration of  $\text{CO}_2$  output produced by the same concentration of this sample of enzyme when tested by the method of Section I. At *pH* 7.6 the uptake of  $\text{CO}_2$  occurs almost entirely through the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ . Fig. 3b, however, gives the uptake of  $\text{CO}_2$  by a solution containing 0.7 *M*  $\text{NaHCO}_3$  and 0.57 *M*  $\text{Na}_2\text{CO}_3$ . The *pH* of this solution being about 10, the reaction

$\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^-$  is also occurring to a marked extent according to Faurholt [1925], confirmed by Brinkman, Margaria and Roughton [1933]. One part of crude  $\text{CHCl}_3$  enzyme in 40,000 parts of solution accelerates in this case, but to a less extent, viz. 1.7-fold.

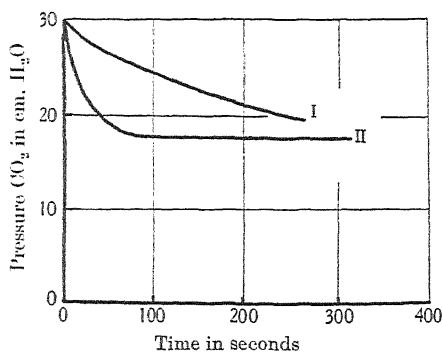


Fig. 3a. I=Rate of uptake of  $\text{CO}_2$  by 0.2 *M* phosphate buffer pH 7.6; II=Rate of uptake of  $\text{CO}_2$  by 0.2 *M* phosphate buffer pH 7.6, with crude  $\text{CHCl}_3$  enzyme 1 in 40,000 added. Temp. 17° C.

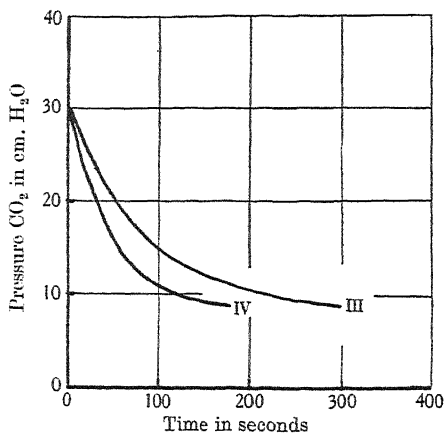


Fig. 3b. III=Rate of uptake of  $\text{CO}_2$  by 0.71 *M*  $\text{NaHCO}_3$ —0.57 *M*  $\text{Na}_2\text{CO}_3$ ; IV=Rate of uptake of  $\text{CO}_2$  by 0.71 *M*  $\text{NaHCO}_3$ —0.57 *M*  $\text{Na}_2\text{CO}_3$ , with crude  $\text{CHCl}_3$  enzyme 1 in 40,000 added. Temp. 17° C.

The enzyme can also be shown to catalyse  $\text{CO}_2$  uptake by pH 11 solutions of  $\text{Na}_2\text{CO}_3$ , in which case the uncatalysed process occurs almost entirely *via* the reaction  $\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^-$ .

*Some more composite processes catalysed by carbonic anhydrase.*

Any complex process, in which the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$  or  $\text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^-$  is a link and is a limiting factor in the over-all speed of the whole, should be accelerated by carbonic anhydrase. Four cases of this kind were investigated, all of possible physiological bearing.

(a) *The solution of  $\text{CaCO}_3$  by weak acids other than carbonic acid, i.e.  $\text{CaCO}_3 + 2\text{HA} \rightarrow \text{CaA}_2 + \text{H}_2\text{CO}_3$ , followed by  $\text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O}$ .* If the chalk is shaken violently enough, the reaction  $\text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O}$  tends to become a limiting factor.

A 2 p.c. suspension of  $\text{CaCO}_3$  in water was shaken at  $0^\circ \text{C}$ . with an equal volume of acetate buffer *pH* circa 5, total acetate conc. 0.7 *M*. One part of crude  $\text{CHCl}_3$  enzyme per 10,000 parts of suspension accelerated the solution of  $\text{CaCO}_3$  three times.

(b) *The solution of  $\text{CaCO}_3$  by carbonic acid to form calcium bicarbonate.* Exp.: 20 c.c. of saturated  $\text{Ca}(\text{OH})_2$  were shaken in a 70 c.c. boat with pure  $\text{CO}_2$  at  $1\frac{1}{2}$  atmospheres initially.

The uptake of  $\text{CO}_2$  was followed manometrically. There was at first a rapid uptake of  $\text{CO}_2$  occasioned by conversion of the  $\text{Ca}(\text{OH})_2$  into  $\text{CaCO}_3$  with precipitation of the latter. This was followed by a smaller slow uptake due to the partial conversion of insoluble  $\text{CaCO}_3$  into slightly soluble  $\text{Ca}(\text{HCO}_3)_2$ , i.e.  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ , followed by  $\text{H}_2\text{CO}_3 + \text{CaCO}_3 \rightarrow \text{Ca}(\text{HCO}_3)_2$ .

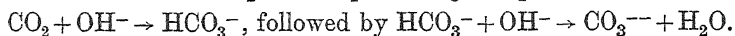
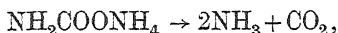
That the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$  is to some extent limiting was shown by addition of crude  $\text{CHCl}_3$  enzyme 1 in 16,000.

The slow process was then accelerated about twelve times at  $0^\circ \text{C}$ .

The rates of solution and deposition of  $\text{CaCO}_3$  are of biological interest in regard to the formation of shells and other calcareous deposits.

(c) *The uptake of  $\text{CO}_2$  by ammonia solution.* This was also measured manometrically. There is first of all a very rapid uptake of  $\text{CO}_2$ , due to the formation of ammonium carbamate, i.e.  $2\text{NH}_3 + \text{CO}_2 \rightarrow \text{NH}_2\text{COONH}_4$ . This is followed by a long slow process in which ammonium carbonate and bicarbonate are formed, the final proportions of carbamate, carbonate and bicarbonate depending upon the initial amounts of  $\text{NH}_3$  and  $\text{CO}_2$ . Of these two processes, the rapid one, i.e. the carbamate formation, does not seem to be affected by carbonic anhydrase, but the slow one is much speeded up. Thus with 4 *M* ammonia, 1 part of crude  $\text{CHCl}_3$  enzyme in 6000 parts accelerated the slow process ten times and even in "Ammon. Fort." (sp. gr. 0.880) the enzyme still showed appreciable activity. This is a striking instance of its robustness.

(d) If an equilibrium mixture of ammonium carbamate, carbonate and bicarbonate is shaken with  $\text{CaSO}_4$  suspension, the carbonate and bicarbonate react very quickly with the  $\text{CaSO}_4$  to form  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{CaCO}_3$  (which is precipitated): calcium carbamate is, however, relatively soluble and does not precipitate, but with the removal of the carbonate from the sphere of action, the carbamate changes over slowly into carbonate which then precipitates. Faurholt [1925] has shown that the conversion of ammonium carbamate into bicarbonate and carbonate occurs, not by a simple addition of water as has often been thought, but by a two stage process.



If so, then the precipitation of  $\text{CaCO}_3$  in the slow stage should be accelerated by carbonic anhydrase. This was confirmed by estimating the amount of  $\text{CaCO}_3$  precipitate formed after various periods of shaking. In the slow stage 1 part crude  $\text{CHCl}_3$  enzyme in 1000 parts accelerated the process about 3.5 times at  $19^\circ \text{C}$ .

Examples (c) and (d) are of physiological bearing in regard to the rôle of carbonic anhydrase in the interaction of carbamates and bicarbonates. In our adjoining paper [1933 b] we give new evidence for the existence of protein-carbamate compounds in physiological fluids especially at low temperatures.

*Some notes on the kinetics of the enzyme.*

Only preliminary observations have been made so far upon the effect of varying enzyme concentration, substrate concentration, temperature, pH and neutral salts upon the kinetics of the reaction.

(a) *Enzyme concentration.* All the data so far available have been given in Section I.

(b) *Substrate concentration.* Experiments have been done upon the effect of varying the pressure of  $\text{CO}_2$  upon the rate of  $\text{CO}_2$  uptake by  $0.2 M$  phosphate buffer pH 7.6, and by  $0.7 M \text{NaHCO}_3 + 0.57 M \text{Na}_2\text{CO}_3$  pH circa 10. In Fig. 4 the rate is plotted against  $\text{CO}_2$  pressure both for the uncatalysed and the catalysed reaction in both cases. For the catalysed experiments 1 part crude solid enzyme per 40,000 parts solution was used at both pH's. From 20 mm. Hg to 380 mm. Hg the rate of the catalysed reaction is proportional to the  $\text{CO}_2$  pressure, but falls off at higher pressure in the pH 7.6 case. A similar result was found for rate of uptake of  $\text{CO}_2$  by  $4 M \text{NH}_3$  almost saturated with  $\text{NaCl}$ ; in this

case, owing to the depressing action of the strong NaCl, 1 part of the same crude solid enzyme per 800 parts of solution had to be used. This indicates that the Michaelis constant of the enzyme [see Haldane, *Enzymes*, 1930] is small, both in conditions where the activity of the enzyme is high, and also where the activity is low.

The uptake of  $\text{CO}_2$  is a far more suitable case for studying the effect of varying the concentration of substrate than is the output of  $\text{CO}_2$ , for with the boat technique the pressure of  $\text{CO}_2$  can be varied from 10 mm. Hg to 1500 mm. (*i.e.* 150-fold), whereas the concentration of  $\text{NaHCO}_3$

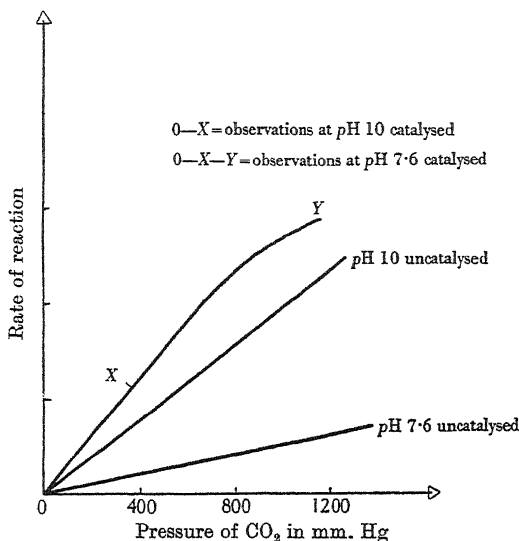


Fig. 4. Relation between substrate concentration and accelerating effect of enzyme.

cannot be conveniently varied more than about fifteen-fold. Furthermore, in the latter case with high concentrations of  $\text{NaHCO}_3$  activity effects and other "neutral salt" effects, cause serious disturbances, but with high pressures of  $\text{CO}_2$  in uptake experiments these troubles do not arise.

Only one experiment has so far been done on  $\text{CO}_2$  output with varying substrate concentration. In this it was found that 1 part crude  $\text{CHCl}_3$  enzyme in 8000 accelerated  $\text{CO}_2$  output from 1.0 M  $\text{NaHCO}_3$  + 1.0 M phosphate buffer pH 6.8 three times, whereas 1 part of the same enzyme in 40,000 accelerated  $\text{CO}_2$  output from the usual 0.2 M  $\text{NaHCO}_3$  + 0.2 M phosphate ten times.

(c) *Effect of temperature.* The rate of  $\text{CO}_2$  output by the standard technique of Section I at  $0.2^\circ\text{C}$ . and  $19.2^\circ\text{C}$ . showed a temperature coefficient per  $10^\circ\text{C}$ . of 2.1 for the catalysed part of the reaction, the enzyme used being a purified  $\text{Ca}_3(\text{PO}_4)_2$  preparation. Much further work is needed here also.

(d) *Effect of pH.* This has not yet been studied systematically, but the impression gained is that the enzyme has an optimum in the physiological range. For  $\text{CO}_2$  uptake experiments above pH 11, and for output experiments below pH 5.5, it will be necessary to resort to Hartridge and Roughton's rapid reaction methods, or perhaps to some variant of the processes discussed under the heading "Scope of enzyme action" in this section.

(e) *Neutral salts.* High concentrations of salt, especially NaCl, have been found to exert a very depressing effect upon the enzyme activity both in  $\text{CO}_2$  output and  $\text{CO}_2$  uptake experiments.

(f) *A special advantage of carbonic anhydrase for kinetic studies.* Brinkman, Margaria and Roughton [1933] have measured the rates of the reactions  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$  under the conditions where the time for the reaction to be half completed ranged from 15 min. to 0.01 sec., i.e. a 100,000-fold variation. These same methods are applicable to the catalysed reaction, which it is therefore hoped to follow over a much wider range of enzyme concentration than has been tried in the case of any other enzyme reaction.

## SECTION V. THE ACTION OF VARIOUS INHIBITORS, AND POSSIBLE ACTIVATORS.

The substances which have been tried may be divided into four classes:

(a) Those which might act by poisoning some active centre in the enzyme of a metallic type (e.g. a hæmatin, iron or copper nucleus), viz. carbon monoxide (in dark and in light), cyanides, sulphides, azides, pyrophosphates.

(b) Other anions which have been shown to inhibit particular enzyme systems, viz. iodoacetate, fluoride.

(c) An extensive series of metallic salts, representative of most of the groups of the periodic table.

(d) Miscellaneous substances.

The general method used for testing the activity of the enzyme in presence of these various substances has been that described in Section I.



*Classes (a) and (b).*

*Carbon monoxide.* The boat technique was used with the following modification: the phosphate solution and bicarbonate solution, with enzyme contained in one or other of them, were saturated with pure CO in Thunberg tubes. Controls in which the enzyme was added subsequently showed that this shaking did not itself cause inactivation of the enzyme.

2 c.c. of each CO saturated reagent were then placed in the boat, the air in the latter replaced by pure CO, and after temperature equilibration the boat was shaken and CO<sub>2</sub> output followed in the ordinary way. The CO was prepared by acting on sodium formate with conc. H<sub>2</sub>SO<sub>4</sub> and was stored under pressure over alkaline sodium hydrosulphite. It thus contained no O<sub>2</sub> or CO<sub>2</sub>. Controls in which hydrogen was stored over alkaline sodium hydrosulphite under the same conditions showed that the gas did not thereby acquire any traces of inhibitory substances (*e.g.* SO<sub>2</sub>, H<sub>2</sub>S or particles of liquid spray). For experiments in the dark the water in the bath was replaced by a deeply coloured dye solution, whilst for light experiments a mirror was placed at the bottom of the bath, and a 100 watt lamp was placed just above the surface of the clean water in the bath.

The results of some experiments are given in Table II:

TABLE II.

Nature of enzyme preparation	Date	Activity expressed in p.c. of activity in air		In CO dark
		In CO dark	In CO light	In CO light
Dilute human blood	29. iv. 32	—	—	80.0 p.c.
Crude CHCl <sub>3</sub> enzyme	16. ix. 32	36.0	32.0	112.0
Al(OH) <sub>3</sub> purified enzyme	20. iv. 32	36.0	70.0	53.0
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> purified enzyme	23. vi. 32	56.1	59.5	94.5
" " "	22. viii. 32	23.3	30.5	77.0
" " "	29. vi. 32	25.0	22.7	110.0

In all instances carbon monoxide produced a large inhibition in the dark; in half the cases, *viz.* the crude blood, the Al(OH)<sub>3</sub> preparation and one of the Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> preparations, this inhibition was partially reversed by light, though in the other cases the light rate and the dark rate in CO were almost the same within experimental error. In none of the four purified preparations was there any visible trace of hæmatin present, but all these showed marked inhibition by CO in the dark, and in two cases this inhibition was partially reversed by light. Further work,

however, is needed to discover why the effect of light is variable, and in positive cases to investigate its action in detail.

*Cyanides.* The action of HCN (prepared by neutralization of KCN to  $pH\ 7.4$ ) was studied upon crude blood and upon the enzyme in the various stages of purification described in Section II, methods *B* and *C*. The effect of various concentrations, ranging from  $M/800$  to  $M/16,000$  HCN, upon a  $Ca_3(PO_4)_2$  purified enzyme solution, is shown in Fig. 5. Practically complete inhibition is produced by  $M/800$  HCN. The latter

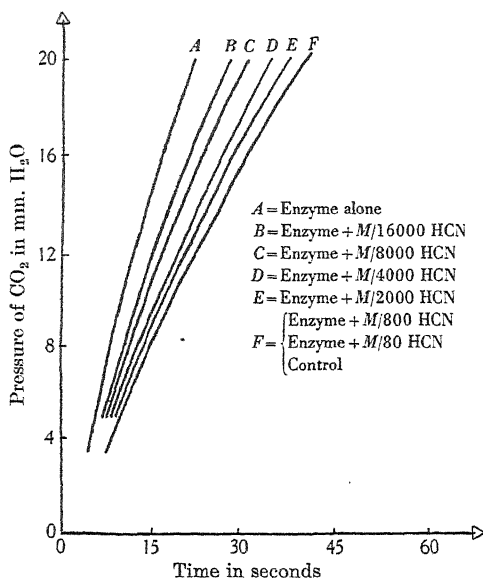


Fig. 5. Effect of various concentrations of HCN upon activity of enzyme.

was also found to be the case with four different samples of crude ox blood, one sample each of horse blood and whale blood, and one sample of enzyme purified by method *B* of Section II.

Considerable inhibition by  $M/4000$  HCN was noted in the case of one sample of  $Al(OH)_3$  purified enzyme, including the ultra-filtrate from the latter through a 6 p.c. Bechhold membrane, and also in the case of one sample of human blood. These two cases were not tested with  $M/800$  HCN.

At the outset of our research we met with a solution of electro-dialysed ox hæmoglobin, which showed slight activation by  $M/1000$  HCN instead of inhibition. Brinkman [1932] also has met with such cases.

Unfortunately we threw away all our material before we realized its value, and since then we have met with no other such case, though we have been continually on the look-out. The detailed investigation of an anomalous sample of such a kind might well throw important light on the nature of the enzyme.

*Sulphides.* The same group of enzyme preparations as those studied for cyanide inhibition (with the exception of human and whale bloods and the  $\text{Ca}_3(\text{PO}_4)_2$  purified enzyme, none of which were tested at all) also showed complete inhibition by  $M/1000 \text{ Na}_2\text{S}$ . At lower concentrations than  $M/1000$ , sulphide appears to be a somewhat more effective inhibitor than cyanide. Sodium sulphide was also shown to inhibit the catalysis of  $\text{CO}_2$  uptake by  $4.0 M$  aqueous ammonia.

*Sodium azide* ( $\text{NaN}_3$ ). A  $M/500$  solution of this reagent was tested on one sample of  $\text{Al}(\text{OH})_3$  purified enzyme and caused a partial inhibition.

*Pyrophosphate, sodium fluoride* ( $M/1000$ ), *sodium iodoacetate* ( $M/1000$ ), were all negative as regards inhibition.

#### Class (c). *Metallic salts.*

Salts of twenty-three different metals were tried, the final concentration in most cases being  $M/1000$  or  $M/2000$ . A  $\text{Ca}_3(\text{PO}_4)_2$  purified enzyme preparation was used in every case except Na, K and Mg. In the latter a crude  $\text{CHCl}_3$  preparation was used. Negative results were found in the following groups of the periodic table.

- Group 1 A. Na, K.
- Group 2 A. Be (as sulphate), Mg (as sulphate), Ca.
- Group 2 B. Cd (as sulphate and as chloride).
- Group 3 A. Ce (as chloride).
- Group 3 B. Al.
- Group 4 A. Ti (as chloride).
- Group 4 B. Pb (as nitrate).
- Group 6 A. Cr (as chloride).
- Group 7 A. Mn.
- Group 8. Fe (as ferrous sulphate + ferric chloride), Co (as nitrate), Ni (as chloride),  $\text{H}_2\text{PtCl}_6$ .

Positive results were found for:

- |  |                          |
|--|--------------------------|
| Group 1 B. Cu (as $0.0005 M$ $\text{CuSO}_4$ recrystallized)     | About 50 p.c. inhibition |
| Ag (as $0.001 M$ nitrate)  | Large inhibition         |
| Au (as $0.001 M$ $\text{HAuCl}_4$ )                              | " "                      |
| Group 2 B. Zn (as $0.001 M$ $\text{ZnSO}_4$ not recrystallized)  | About 50 p.c. inhibition |
| Hg (as $0.0005 M$ $\text{HgCl}_2$ recrystallized twice)          | Large inhibition         |
| Group 5 A. Va (as $0.001 M$ vanadyl sulphate not recrystallized) | " "                      |

With the exception of vanadium, metals which act as poisons to carbonic anhydrase are those of Groups 1 B and 2 B only. Such also have

been observed for a number of other enzymes, viz. xanthine oxidase [Wieland and Mitchell, 1932], saccharase [Mÿrbäch, 1926], papain, kathepsin [Krebs, 1930], and may indeed be an empirical principle of wide application for enzymes. The present case, however, cannot yet be quoted as a satisfactory example, because the reagents used in testing the enzyme activity, viz. phosphate and bicarbonate at pH 9.2, both form precipitates with many of the metals tested. Some of the inactive metals may have been ineffective on account of this purely inorganic factor, and we cannot be sure that if it were controlled there would any longer be such a sharp demarcation between them and the active group Cu, Ag, Au, Zn and Hg.

Such control would be very difficult to effect. At all events there is no doubt that Cu, Ag, Au, Zn and Hg are strong poisons for carbonic anhydrase.

*Class (d). Some other substances.*

*Indifferent gases:*  $N_2$ ,  $O_2$ ,  $H_2$ . The activity of the crude  $CHCl_3$  enzyme in nitrogen, oxygen and hydrogen was found to be the same as in air.

*Phenylurethane.* This was chosen as an example of a commonly used cell narcotic. The phosphate and bicarbonate solutions were saturated with phenylurethane by shaking in Thunberg tubes for an hour or more and were then allowed to stand. Horse hæmoglobin, crude  $CHCl_3$  enzyme, and  $Ca_3(PO_4)_2$  purified enzyme all showed about the same inhibition, i.e. roughly 50 p.c.

*Saponin.* A 4 p.c. solution of ox blood dissolved in 1 p.c. saponin showed the same activity as a 4 p.c. solution of the same ox blood in water.

*Hydrazine sulphate.* A 0.002 *M* solution had no effect upon the activity of a crude  $CHCl_3$  preparation.

*Ferricyanide.* Blood dissolved in 0.001 *M*  $K_3FeCy_6$  has the same activity as blood dissolved in water.

## SECTION VI. THE DISTRIBUTION OF THE ENZYME AND SOME PHYSIOLOGICAL CONSIDERATIONS.

### *Distribution.*

During this work, many estimations of the enzyme content of goat, ox and human blood have been made. These are collected in Table III, which also gives the content of some other mammalian bloods in single instances.

TABLE III.

Animal	No. of cases	E/c.mm. blood	Average E/c.mm. blood
Goat (anæsthetized)	11	1.30-6.10	2.80
Ox (defibrinated)	6	0.60-1.80	1.10
Man	8	0.37-0.68	0.55
Rabbit	2	0.71-1.72	1.21
Goat (normal)	1	1.35	1.35
Horse	1	0.64	0.64
Rat	1	1.70	1.70
Whale	1	1.40	1.40

The values were the same, whether clotting was prevented by defibrination or by oxalate. In any one species there is a two- to four-fold variation or more in normal animals; this suggests that the normal carbonic anhydrase content of mammalian blood is abundantly in excess of minimum physiological requirements. It seems doubtful whether there is any significance in the higher average content of goat's blood as compared with ox, and man.

During the last four months of this research we were lucky enough to have the opportunity, through the kind offices of Prof. Barcroft and his colleagues, of studying an exceptionally interesting case of distribution.

About a dozen female goats were "stocked" during the latter part of January 1933 and were operated on in roughly successive weeks from March 20 to June 1. Samples of the foetal blood and maternal blood (treated with oxalate and fluoride) were given to us in each case and the carbonic anhydrase content of each estimated. The results are shown in Table IV, together with a single case of whale mother and foetus, the blood of which was kindly supplied by Mr A. Laurie.

TABLE IV.

Date of operation	Date stocked	E/c.mm. mother blood	E/c.mm. foetal blood	Activity of foetal blood expressed in p.c. of activity of mother blood
Mar. 23	Feb. 2	1.6	<0.010	<0.5
April 11	Jan. 30	3.6	0.022	0.6
" 13	" 24	4.2	0.140	3.3
" 18	" 23	1.8	0.060	3.4
" 24	" 21	6.1	0.110	1.8
May 1	" 20	1.3	0.067	5.2
" 5	" 19	1.6	0.056	3.5
" 12	" 18	1.9	0.066	3.5
" 22	" 16	1.7	0.040	2.3
" 31	" 12	5.0	0.456	9.2
June 1	Unknown, probably 15 weeks $\pm$	1.9	0.292	15.0
May 31	Whale foetus of unknown age	1.4	0.350	25.0

In the very young foetuses there is extraordinarily little enzyme and the amount does not begin to rise appreciably till very near the end of term. Since the foetuses live in an all fluid environment there is perhaps not so much need for the enzyme, since it is possible for them to excrete  $\text{HCO}_3^-$  into the mother blood as well as  $\text{CO}_2$ . It is, however, indispensable for them to have a good supply before parturition occurs, otherwise when they begin to breathe they may not be able to excrete  $\text{CO}_2$  gas into their expired air fast enough. The consequences of the low content of enzyme in foetal blood must indeed be taken fully into account in working out quantitatively the gas exchange between maternal and foetal blood in the placenta. Finally, since the hæmoglobin content of the foetal blood was never less than 50 p.c. of that of the maternal blood, this series of experiments gives a clarion instance of the independence of the enzyme from hæmoglobin.

The distribution of the enzyme in the blood of lower organisms, *e.g.* earthworm, planorbis, etc., was reported in the preliminary communication of Brinkman, Margaria, Meldrum and Roughton [1932]. The measurements of activity were made by a portable, but much less accurate, modification of the boat technique, and are too rough for values of  $E/c.\text{mm.}$  to be worth calculating.

Tests on some other materials besides blood have been made with the following results:

*Cow's milk, normal human urine.* Both inactive even when 1 part in 10 is added to the phosphate solution in the boat test.

*Rabbit's sperm.* Showed a content of 0.0034  $E/c.\text{mm.}$  This sperm was kindly supplied by Dr J. Walton and appeared to be quite free from blood.

*Muscle extract.* Water extracts of the hind limbs both of a dog and a cat were made, after the muscles had been perfused with isotonic saline until the issuing fluid was almost free of blood hæmoglobin. In both cases an activity was observed, and it was found that the enzyme activity of the muscle extract/content in muscle hæmoglobin (measured colorimetrically) was roughly equal to the enzyme content of the blood/hæmoglobin content of the blood of the same animal.

*Plasma.* No carbonic anhydrase could be detected in oxalated rabbit's plasma even when tested 1 part in 10, provided that allowance was made for the very slight amount of laking of the red corpuscles which occurred before the plasma was separated. This is confirmed by the experiments with unlaked goat's blood described below.

*Yeast.* No enzyme could be detected in an extract of 15 g. Belgian top yeast ground with 100 c.c. of a mixture of  $M/5 \text{ Na}_2\text{HPO}_4$  and  $M/5$

$K_2HPO_4$  and allowed to stand for 24 hours at  $1^\circ C.$  before being centrifuged.

In one experiment with a zymine preparation a slight positive effect was noticed. This needs confirmation. No carbonic anhydrase has yet been found in green plant material.

*Comparison between laked and unlaked blood.*

In early experiments, confirmed recently, with defibrinated ox blood, obtained from the butcher, we noticed that the activity of unlaked blood was about 23–35 p.c. of that of the same amount of laked blood. This was also true if the unlaked blood corpuscles were washed three times with saline to eliminate the slight laking which is almost always present in defibrinated blood. We attributed the lower activity of the unlaked corpuscles simply to the fact that the substrate has to diffuse into the corpuscles before it can react with the enzyme, so that this is another case of chemical reaction velocity and diffusion being joint limiting factors [Roughton, 1932].

We were, therefore, surprised to find that in goat's blood, drawn in the laboratory by syringe from a vein, the activity of unlaked corpuscle suspensions was, in all cases but one, only 0.05–0.01 p.c. of the activity of the same amount of laked blood, when tested by the method of Section I. In each case the blood was put in the 0.2 *M* phosphate, and the result was the same whether the goat was anaesthetized or not and independent of whether clotting was stopped by defibrination or by oxalate-fluoride solution.

The fact that whole blood was used for these "unlaked" experiments also confirms that plasma and serum are both practically inactive. The very slight activities observed may indeed be due to traces of laking.

The result must apparently be a permeability effect of the corpuscle membrane, which may depend upon the species of animal, the manner in which the blood is drawn, and the medium in which it is suspended. In any case, much further work on these lines is needed not only to clear up the subject for its own sake, but also to enable us to assess the activity of carbonic anhydrase in circulating blood *in vivo*.

*Possible rôle of the enzyme in other processes than evolution of  $CO_2$  in the lung.*

Carbonic anhydrase may possibly have some effect in earlier stages of the metabolism, than the final one in which  $CO_2$  is evolved from the body. The only one so far tested is the fermentation of glucose-phosphate

mixture pH 7.6 by zymine at 26° C. No effect was observed. Many other possibilities, such as decarboxylation, formation of urea in the liver, effect in muscular activity, etc., need to be explored, as do also the effects of injection of the purified enzyme. Brinkman [private communication] tells us that he has made a start at the latter problem. Further work, on such lines, is contemplated in collaboration with him and his co-workers.

#### SUMMARY.

1. Carbonic anhydrase is an enzyme present in red blood corpuscles (but not in the blood plasma) which catalyses both phases of the reversible reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$ . It is thus of prime physiological importance in the formation of  $\text{CO}_2$  from bicarbonate in the lung. Without it,  $\text{CO}_2$  could not be excreted nearly fast enough for the needs of the body.

2. The activity of the enzyme can be estimated by its accelerating effect upon the evolution of  $\text{CO}_2$  from a mixture of 0.2 *M* phosphate buffer pH 6.8 with 0.2 *M* bicarbonate. At low concentrations of enzyme, the acceleration is directly proportional to the amount of enzyme added.

3. The enzyme can be separated from the hæmoglobin of the red blood corpuscles by coagulating the hæmoglobin. Three different methods were tried with success. In the best of these, ox corpuscles are treated with an equal volume of 40 p.c. alcohol, and then with half their volume of chloroform. The mixture is then shaken and centrifuged. The supernatant fluid is practically colourless and contains roughly 50 p.c. of the total enzyme of the blood. This crude preparation is further purified by adsorption with  $\text{Al}(\text{OH})_3$  cream or  $\text{Ca}_3(\text{PO}_4)_2$  suspension and by ultra-filtration. The purest product is about 1800 times as active as the original blood and seems to be free from hæmoglobin, hæmatin, catalase, peroxidase and oxidase. The enzyme is very stable when evaporated to a solid.

4. The enzyme is stable in solution over the range pH 3–12. Its solution in water is destroyed by 30 min. heating at 65° C. It shows most of the protein reactions. It accelerates a number of processes with over-all velocity dependent upon the rate of one or other of the reactions  $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ , or  $\text{CO}_2 + \text{OH}^- \rightleftharpoons \text{HCO}_3^-$ .

5. The enzyme is poisoned by CO, cyanides, sulphides, azides, Cu, Ag, Au, Zn, Hg, phenylurethane. A number of other substances were tested, but had no effect.



6. Preliminary work has been done on the kinetics of the enzyme action, and the effect of temperature, salts and  $pH$  thereon.

7. The foetal blood of goats has far less enzyme than the maternal blood, but the content rises towards the end of gestation. A suspension of unlaked corpuscles is much less active than a solution of laked corpuscles.

8. Some other instances of the distribution of the enzyme are given.

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## THE STATE OF CARBON DIOXIDE IN BLOOD.

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## INTRODUCTION.

In another paper in the present *Journal* we [Meldrum and Roughton, 1933 *b*] have described the isolation from blood corpuscles of the enzyme carbonic anhydrase, which catalyses both phases of the reversible reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$ . In the introduction to that paper and elsewhere we pointed out that it is only through the presence of this enzyme that such  $\text{CO}_2$  as is carried as bicarbonate in the blood can be transported to the expired air fast enough for the needs of the body. If all the carbonic anhydrase is either removed from the blood, or is prevented from acting by the addition of some poison, *e.g.* cyanide, then the change from  $\text{CO}_2$  to bicarbonate and *vice versa* must become relatively slow, and any rapid chemical processes still occurring between such "mutilated" blood and  $\text{CO}_2$  must be due to some other chemical reaction.

We shall begin by describing the rate of  $\text{CO}_2$  output from, and  $\text{CO}_2$  uptake by, cyanide blood as compared with normal blood.

By such means we have confirmed the view of Henriques [1928] that, under suitable circumstances, appreciable amounts of  $\text{CO}_2$  combine rapidly and reversibly with blood to form compounds of some other kind than bicarbonate, and to a greater extent in reduced blood than in oxygenated blood. We next give a series of experiments upon  $\text{CO}_2$  uptake at various *pH*'s by ammonia, certain amino acids and polypeptides, which parallel very closely the experiments upon cyanide treated blood, and hence lead to the conclusion that the  $\text{CO}_2$  compound in the case of blood is of the same type as in the case of the simpler amino compounds, namely of a carbamate type. In the last section of this paper we consider the results of previous workers upon the nature of  $\text{CO}_2$  compounds in blood in the light of this conclusion, and also discuss shortly the physiological importance of carbamates in  $\text{CO}_2$  transport.

SECTION I. CO<sub>2</sub> OUTPUT EXPERIMENTS ON NORMAL AND ON CYANIDE-POISONED BLOOD.

To begin with, it will be useful to recall Henriques' [1928] experiments in some detail. Hæmoglobin solutions or serum were equilibrated with various CO<sub>2</sub> pressures, and 2 c.c. samples of such solutions were then introduced suddenly into the evacuated chamber of a modified van Slyke gasometric apparatus. Immediately afterwards the apparatus was shaken for a known period, at the end of which the shaker was stopped and the amount of CO<sub>2</sub>, which had escaped into the gas phase, was measured by the usual methods. A series of such experiments

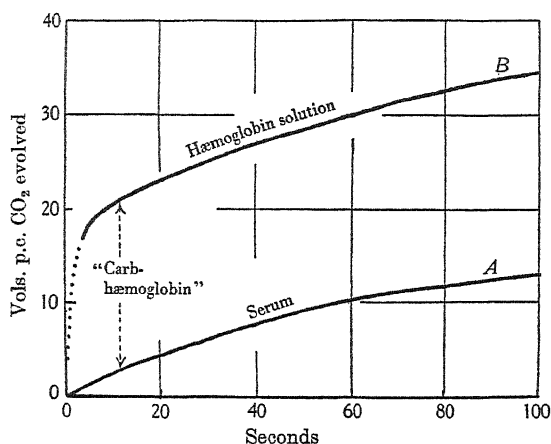


Fig. 1. Rate of evolution of CO<sub>2</sub> from hæmoglobin solutions and from serum as studied by Henriques.

with shaking periods of 5 sec. and upwards (to 10 min.) gave the curve of CO<sub>2</sub> evolution plotted against time.

In the case of horse serum the curves were of the type shown in Fig. 1, *A*, i.e. were similar to those of Brinkman, Margaria and Roughton [1933] for the rate of CO<sub>2</sub> evolution from mixtures of bicarbonate and phosphate buffer, and indeed the actual rate of CO<sub>2</sub> evolution from serum was of the order to be expected from the velocity constants of the reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$  in absence of catalyst.

In the case of horse hæmoglobin solutions, however, the curves were of a different type (Fig. 1, *B*). A certain proportion (in some cases as much as 40 p.c.) of the CO<sub>2</sub> came off very suddenly in the first 5 sec., whereas the remainder of the CO<sub>2</sub> came off in the same gradual manner,

and at about the same rate as, from serum or from phosphate-bicarbonate mixtures of that pH. Henriques thought that the sudden rapid evolution of  $\text{CO}_2$  could not be explained by the presence in his hæmoglobin solutions of a catalyst for the  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}_2\text{O} + \text{CO}_2$  reaction, for there seemed no conceivable reason why such a catalyst should only act for 5 sec. and then stop acting. He attributed it, on the other hand, to the rapid dissociation of a reversible compound between  $\text{CO}_2$  and hæmoglobin, which he called "carb hæmoglobin."

These results were very mystifying to us, in view of the large amounts of carbonic anhydrase present in blood, and of the fact that hæmoglobin solutions prepared by the ordinary methods have so far proved to be richly contaminated with this enzyme.

We started then by repeating some of Henriques' experiments with a more labour-saving technique which enabled us to get the whole curve for the  $\text{CO}_2$  evolution on one sample of solution in a single experiment:

2 or 4 c.c. of ox blood, laked by freezing and thawing, was equilibrated thoroughly with air containing 50 mm. Hg pressure of  $\text{CO}_2$ , and then introduced suddenly into a glass boat-shaped vessel (capacity 72 c.c.), mounted in a shaker in a constant temperature water bath. The boat was then at once closed by a stopper, a lead through which connected with the manometer, and the shaker started as soon as possible. From readings of the manometer at 5, 10, 15, 30 sec., etc., the curve of  $\text{CO}_2$  evolution was plotted. This technique is thus a particular example of the "boat" method of Brinkman, Margaria and Roughton [1933], used by them in studying the kinetics of the uncatalysed  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$  reaction.

The results with normal laked blood, whether of ox or horse, were always of the type shown in Fig. 2, C. There was no evidence of the curve consisting of two phases, one rapid, the other slow; on the other hand the  $\text{CO}_2$  evolution proceeded smoothly to completion in about 60–90 sec. It was indeed just what we expected, for the laked blood contained abundant amounts of carbonic anhydrase, and this should have caused the  $\text{CO}_2$  to be formed from the bicarbonate of the blood and evolved into the gas phase as fast as diffusion conditions permit. In the boat method, controls have shown that, with watery solutions, diffusion prevents the evolution of  $\text{CO}_2$  into the gas phase being completed in less than 20 sec., no matter how fast the antecedent chemical formation of dissolved  $\text{CO}_2$  takes place. It is not surprising that with a much more viscous medium-laked blood the time should be about thrice as long.

The only explanation of Henriques' results, which we could think of, was that in some way or other the carbonic anhydrase in his hæmoglobin solutions had got eliminated or else poisoned.

To test this idea, we repeated one experiment with the addition to the blood of 0.1 *M* HCN (prepared by neutralizing KCN with acetic acid to *pH* 7.4).

Experiments to be described later show that this amount of cyanide is needed to poison the carbonic anhydrase in whole blood completely.

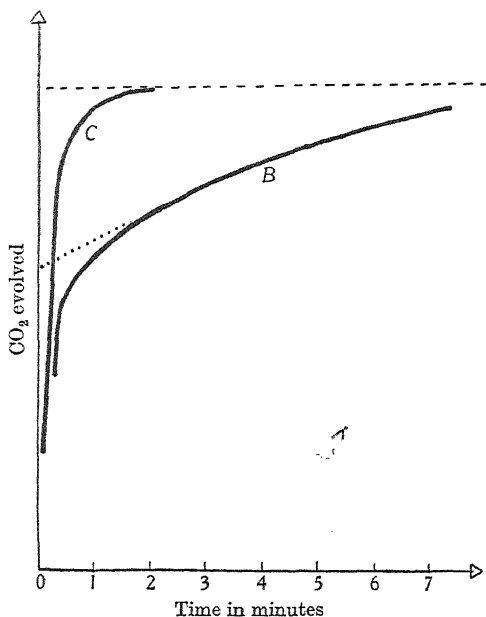


Fig. 2. Effect of poisoning carbonic anhydrase upon rate of evolution of CO<sub>2</sub> from blood. *B*. Blood shaken with 50 mm. CO<sub>2</sub>, then 0.1 *M* HCN added; *C*. Blood shaken with 50 mm. CO<sub>2</sub>, then 0.1 *M* NaCl added; then shaken with CO<sub>2</sub> free gas phase at 0° C.

The curve now obtained, Fig. 2, *B*, shows the typical diphasic effect of Henriques, a large part of the CO<sub>2</sub> coming off very rapidly and the remainder only gradually. The total amount of CO<sub>2</sub> evolved was very nearly the same as that evolved in a control experiment in which the blood was treated with 0.1 *M* NaCl, so as to make the ionic strength the same as in the cyanide-treated blood.

Several confirmatory experiments with slightly varying technique gave a similar result, so that our explanation of Henriques' observations is probably correct. To clinch the matter it would be necessary

to prepare horse hæmoglobin solution in exactly the same manner as Henriques did. It is hoped that it may shortly be possible to do this in conjunction with Dr Henriques.

At this stage we gave up our experiments upon  $\text{CO}_2$  output of blood, and turned instead to the converse process, namely uptake of  $\text{CO}_2$  by blood initially free (or almost free) of  $\text{CO}_2$ .  $\text{CO}_2$  uptake experiments of this kind do not seem to have been done before, but have several marked advantages over the  $\text{CO}_2$  output experiments of Henriques: (a) As far as the bicarbonate mechanism is concerned the rate of uptake is dependent upon the velocity of the hydration reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ . At physiological  $p\text{H}$  and in absence of carbonic anhydrase the velocity of this process in the forward direction  $= k'(\text{CO}_2)(\text{H}_2\text{O})$  and, until the later stages when the back reaction becomes important, is independent of the buffer power and  $p\text{H}$  of the solution. It will be remembered that Hawkins and van Slyke [1930] criticized Henriques for failing to note the importance of the  $p\text{H}$  and buffer power of the solution in his  $\text{CO}_2$  evolution experiments. (b) The hydration reaction, being one in which two molecules, viz.  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , react together, is scarcely affected by activity coefficient corrections. The dehydration velocity  $\text{H}_2\text{CO}_3 \rightarrow \text{H}_2\text{O} + \text{CO}_2$ , which governs the output of  $\text{CO}_2$  in absence of carbonic anhydrase, is on the other hand very much affected by activities, for its rate is proportional to  $(\text{H}_2\text{CO}_3)$  which, in turn, is always proportional to the product of the hydrogen activity and the bicarbonate activity. There is still great uncertainty as to the value of the activity coefficient corrections in strong protein solutions. (c) Lastly  $\text{CO}_2$  uptake experiments can be carried out not only at physiological  $p\text{H}$ 's, but also at far more alkaline  $p\text{H}$ 's (up to  $p\text{H}$  11). Experiments over a wide range of  $p\text{H}$  have proved very valuable in determining the nature of the  $\text{CO}_2$  compounds formed.

## SECTION II. $\text{CO}_2$ UPTAKE EXPERIMENTS ON NORMAL AND ON CYANIDE-POISONED BLOOD.

### *Technique.*

The details of the experimental technique will be described for the uptake of  $\text{CO}_2$  by reduced laked blood poisoned with cyanide, and the small modifications for the other cases can then be treated very briefly:

(i) *Preparation of  $\text{CO}_2$ -free reduced laked blood.* 40 c.c. of defibrinated ox blood is mixed with 3 c.c. 0.2 *M* acetic acid [for a reason to be given later] and placed in a 1500 c.c. Büchner flask and the latter evacuated

in a water bath, temperature 30–40° C., for 1 min. The connection to the filter pump is then closed, and the flask shaken violently by hand for 1 min. Nitrogen from a commercial cylinder is then admitted to the flask, to bring the pressure up to one atmosphere, and the flask evacuated again for 1 min. and then shaken again for 1 min. The process, mentioned in the last sentence, is repeated nine times, and then repeated five times with purified nitrogen instead of commercial nitrogen. A stock of 10 litres of purified  $N_2$  was made by storing commercial  $N_2$  over alkaline sodium hydrosulphite, prepared according to van Slyke. The flask is left full of pure  $N_2$  at a positive pressure, and inverted so as to seal the cork of the flask from leakage. The flask is placed in a  $-10^\circ$  C. chamber for a few hours. On thawing the flask laking is complete. Blood so treated was found on analysis to contain not more than 1–2 vols. p.c.  $CO_2$ .

(ii) *The HCN solution.* 1.1 M HCN in 1.1 M K-acetate pH 7.1–7.4 was prepared in the same manner as in Section I. 4 c.c. of this are blown by pure  $N_2$  into the Büchner flask and mixed there thoroughly with the reduced laked blood. The dissolved  $O_2$  carried in by the cyanide is negligible. The cyanide blood is then transferred anaerobically to a burette, closed at the top with a rubber stopper through which a lead passed to the pure  $N_2$  stock, and having at the bottom a three-way tap.

The blood is left in the burette with a positive pressure of  $N_2$  on top and 4 c.c. samples are drawn off for each experiment as required.

(iii) *The boat technique (vide Fig. 2).* The boat method was used again. One drop of octyl alcohol is placed in the boat, and the latter closed with a two-holed rubber stopper, one lead through which is temporarily closed, whilst the other is connected to the bottom of the blood burette. The side piece of the three-way tap of the burette is connected *via* a T-piece to the vacuum pump and the pure  $N_2$  supply. The boat is evacuated and washed out with pure  $N_2$  twice through the burette tap: the latter is then turned so that 4 c.c. of blood run into the boat and the traces of blood left in the dead spaces are blown into the boat by turning the tap so as to connect to the  $N_2$  supply.

The boat is left with a positive pressure of  $N_2$ , its connections closed with clips  $C_1$ ,  $C_2$  and it is then placed in the shaker in the water bath.

One lead of the boat (its clip  $C_1$  remaining closed) is connected to the manometer, whilst the other lead of the boat ( $C_2$  also still closed) connects to a T-piece  $T_1$ . The compensating bottle  $B'$  of the boat arrangement is connected as usual by one lead to the other side of the



manometer, whilst the second lead from  $B'$  connects to another T-piece  $T_2$ .

$T_1$  and  $T_2$  are connected together by a piece of rubber tubing with a clip on it ( $C_3$ ). The last lead of  $T_2$  is connected *via* a rubber tube, controlled by clip  $C_6$ , to the vacuum and pure  $N_2$  supply, whilst the

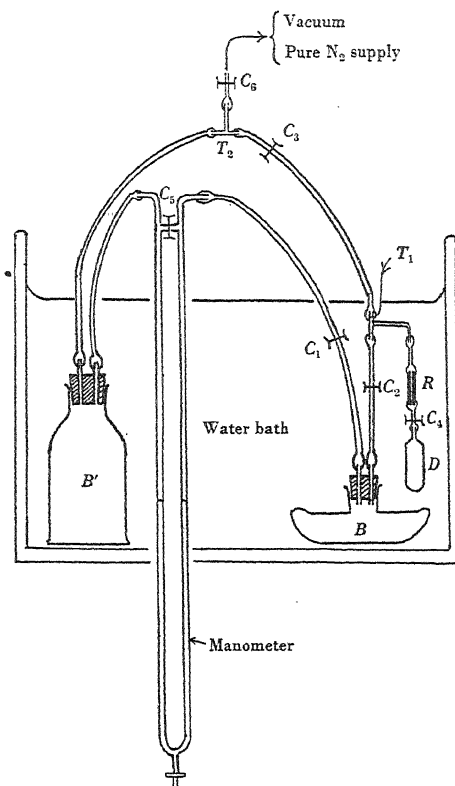


Fig. 3. Arrangement of boat, etc., for  $CO_2$  uptake experiments.

last lead of  $T_1$  is connected to a piece of broken thermometer tubing,  $R$ , joined by pressure tubing (with a clip  $C_4$  on it) to a small vessel  $D$ .  $D$  has been previously filled with  $O_2$  free  $CO_2$ , the gas being first drawn from a commercial  $CO_2$  cylinder into a bottle containing chromous chloride, as used by Warburg, Kubowitz and Christian [1931] for removing  $O_2$  from  $CO_2$  atmospheres. Clip  $C_5$  which controls the connection at the top between the two limbs of the manometer, and clip  $C_3$

being open, the compensating bottle, manometer, and connections up to the closed clips  $C_1$ ,  $C_2$ ,  $C_4$  are now evacuated and washed twice with pure  $N_2$ . Clips  $C_1$ ,  $C_2$  are now opened, and the pressure of  $N_2$  in the whole system reduced to about 80 mm. Hg by evacuation. Clip  $C_6$  is closed and the boat is shaken for 1 min. so as to bring the dissolved gases in the blood into equilibrium with the gas phase, after which the boat is left to rest for 5 min. to allow the films of blood formed during the shaking to drain completely down the sides of the boat.

Clips  $C_3$ ,  $C_5$  are now closed and 25 sec. before shaking is to begin clip  $C_4$  is opened, so that a slow stream of  $CO_2$  issues through the resistance tubing  $R$  into the boat, thereby depressing the level of the gauge liquid on the side of the manometer connected to the boat. The pressure of  $CO_2$  put into the boat is adjusted to a suitable value, this usually taking about 15 sec., the clip  $C_4$  closed and after 10 sec. standing, shaking is begun. During the 10 sec. standing the movement of the manometer is not more than 2 mm. at  $0^\circ C.$  or  $15^\circ C.$  Readings are taken at 5, 10, 15 sec., etc., up to 10 min. or more if necessary and the curve of  $CO_2$  uptake plotted against time. The actual volume of  $CO_2$  taken up by the blood, *i.e.*  $V$  c.c. is given with sufficient accuracy by the formula

$$\frac{p_0 V}{B} = \alpha x (p - W) \left( \frac{1}{B} + \frac{1}{B'} \right) + 2x,$$

where  $B$  = volume of gas phase in boat and connections to manometer;

$B'$  = volume of gas phase in compensating bottle;

$p_0$  = barometer pressure of 760 mm. Hg expressed in cm. manometer liquid;

$p$  = initial pressure of gas in boat and compensating bottle expressed in cm. manometer liquid;

$W$  = water vapour pressure at temperature of bath expressed in cm. manometer liquid;

$\alpha$  = cross-sectional area of manometer in sq. cm.;

$x$  = alteration of level of liquid on one side of manometer caused by the gas exchange (measured in cm.).

For further details, especially as to the limitations of the method, the paper of Brinkman, Margaria and Roughton [1933] should be consulted.

It is advisable in reduced blood experiments to make sure, from time to time, that no contamination with  $O_2$  has occurred at any stage. This may be done by closing clips  $C_1$ ,  $C_2$  at the end of the shaking, and

examining the blood in the closed boat for traces of oxyhæmoglobin by the spectroscope. Such tests were invariably negative in our experiments.

(iv) *Experiments with oxygenated blood.* In this case cylinder  $O_2$  is used instead of  $N_2$  for freeing the blood of  $CO_2$ , and also at every other stage of the process. The evacuation and washing of the various connections, etc., need not be so scrupulous in the case of  $O_2$  experiments as in  $N_2$  experiments.

(v) In the case of normal blood 4 c.c. of water instead of cyanide are run into the blood.

The whole procedure sounds rather complex, but with practice an experiment with reduced blood can be done in about 35 min., and one with oxygenated blood in a few minutes less.

#### *Experiments with normal blood.*

Three experiments were done with defibrinated, laked ox blood at  $0^\circ C.$ , and one with a solution of horse hæmoglobin prepared by washing the corpuscles three times with saline and lysing them with an equal volume of alumina cream, which on centrifuging removed the stroma proteins. The results, in all four cases, were of the type labelled normal blood in Fig. 5. There was again no evidence of two separate phases, one rapid and the other slow;  $CO_2$  uptake proceeded smoothly to completion in 120–180 sec.

#### *Mode of action of cyanide.*

The effect of various amounts of added cyanide is shown graphically in Fig. 4. It will be seen that the curve of  $CO_2$  uptake is slowed down, but remains smooth up to a concentration of  $0.05 M$  cyanide; with  $0.1 M$  cyanide, and  $0.2 M$  cyanide the curve is obviously divisible into two phases. The obvious inference is that in the presence of  $0.1 M$  cyanide, the carbonic anhydrase in the blood is completely poisoned, and the first rapid phase is due to the sum of the  $CO_2$  taken up in physical solution and that taken up in some other chemical form than  $H_2CO_3$  or  $HCO_3^-$ . The  $CO_2$  taken up during the slow phase, however, represents the bicarbonate  $CO_2$ .

That the poisoning of the carbonic anhydrase is complete is shown by the fact that the  $0.2 M$  cyanide curve is identical with the  $0.1 M$  cyanide curve, except that there is a slightly greater rapid uptake in the latter case. This may, however, be explained by the lower solubility of  $CO_2$  in  $0.2 M$  CN blood than in  $0.1 M$  CN blood.

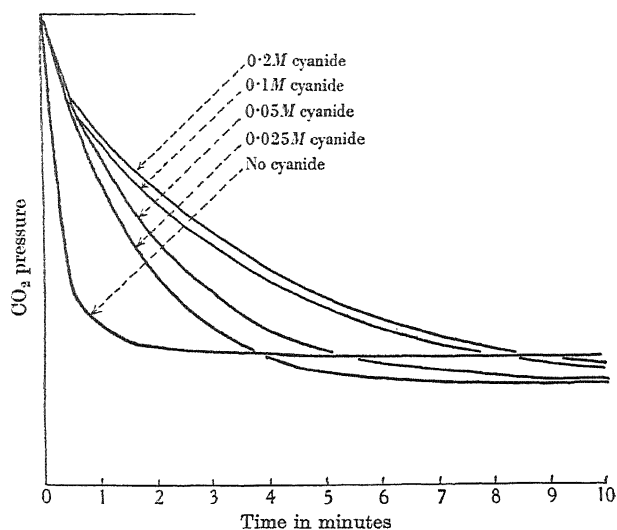


Fig. 4. Effect of various concentrations of cyanide upon rate of uptake of CO<sub>2</sub> by blood at 15° C.

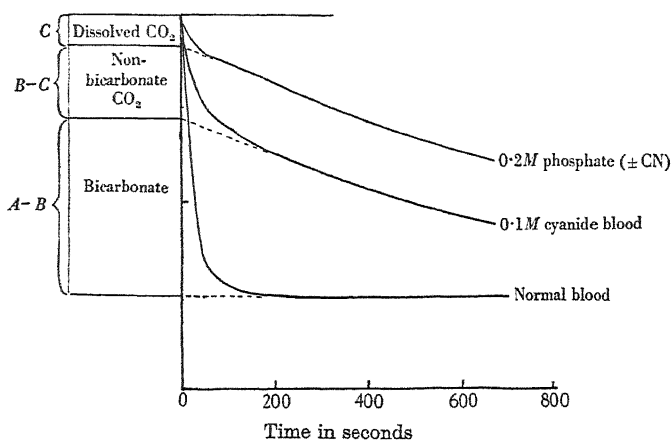


Fig. 5. Method of estimating amount of CO<sub>2</sub> taken up in some chemical, but non-bicarbonate form, during CO<sub>2</sub> uptake by blood.

That the cyanide has no important action on the blood other than the poisoning of the carbonic anhydrase is shown by the following observations and considerations:

(i) The total uptake of  $\text{CO}_2$  by the cyanide blood is practically the same as the total uptake of  $\text{CO}_2$  by normal blood (*vide* Fig. 4). The same is also true of the output of  $\text{CO}_2$  from cyanide blood and normal blood in Section I. Such minor effects as are present may be attributed to difference in ionic strength or *pH*.

(ii) Fig. 5 shows the rate of uptake of  $\text{CO}_2$  by 0.2 *M* phosphate buffer *pH* 7.6, with and without 0.1 *M* cyanide, compared with the rate of uptake of  $\text{CO}_2$  by oxygenated blood treated with 0.1 *M* cyanide. It will be noted that the cyanide has no effect upon the rate of uptake of  $\text{CO}_2$  by phosphate and that once the rapid phase is over in the case of the blood, the slow phase is practically identical in rate and course with the slow phase in the case of phosphate. This is strong evidence that the slow phase in the case of the blood is due to the same cause as in the case of the phosphate, namely the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ , followed by  $\text{H}_2\text{CO}_3 \rightarrow \text{H}^+ + \text{HCO}_3^-$ , with no effective catalyst present for the first of these two reactions.

(iii) As far as is known, cyanide does not act chemically upon oxy-haemoglobin or reduced haemoglobin, though it forms a well-known compound with methaemoglobin. The matter has recently been investigated in detail by Barnard [1933].

Further evidence for this was given by a measurement of the velocity of dissociation of oxyhaemoglobin at *pH* 10, in presence and in absence of 0.1 *M* cyanide, by means of the Hartridge-Roughton [1923] rapid reaction method. The presence of the cyanide was found to have no influence.

This also makes it unlikely that the enzymes present in blood have any direct effect upon the reaction between oxygen and haemoglobin, since the rate of dissociation, which is very sensitive to various influences, is not changed by poisoning of the enzymes of the blood.

One last point arises in connection with the cyanide. The preliminary shaking of the cyanide blood in the boat serves to get the blood and the gas phase of the boat into equilibrium as regards HCN. When the  $\text{CO}_2$  is later taken up by the blood, part of the buffering of it must be done by the cyanide-HCN buffer system. This will give rise to some new HCN in the blood, and part of this will escape into the gas phase and be recorded as a pressure change by the manometer. It can, however, be shown by calculation that only a very slight error will ensue

The relative buffer power of the blood and the cyanide system in most of our experiments was such that about half the fresh hydrogen ions would have been buffered by the cyanide system. At worst then, every mol of  $\text{CO}_2$  taken up could only have given rise to half a mol of HCN in solution in the blood. According to the recent data given in Landolt-Bornstein's tables the partition coefficient of HCN between gas phase and water is at low pressures of HCN at least 1 to 224 at  $18^\circ \text{C}$ . In blood it would be expected to be slightly less.

In most of our experiments the volume of the gas phase/volume of the liquid phase was 17.5.

Therefore for every mol of  $\text{CO}_2$  taken up by the blood not more than  $\frac{17.5}{2 \times 224}$  mols HCN, *i.e.* 0.039 mols, could have escaped into the gas phase. Thus the error could not at most have exceeded 3.9 p.c. at  $18^\circ \text{C}$ ., and at  $0^\circ \text{C}$ ., owing to the increased solubility of HCN, the error must have been considerably less. It will be sufficiently (*i.e.* to within 2 p.c.) accurate therefore if the apparent value of  $\text{CO}_2$  taken up chemically by cyanide blood at room temperature and below be increased by 2 p.c. At higher temperatures, however, a larger and more carefully worked out correction would have to be applied.

*Calculation of amount of rapidly formed  $\text{CO}_2$  compound in cyanide blood.*

Four staple initial pressures of  $\text{CO}_2$  were used in our experiments, *viz.* of 20 cm.  $\text{H}_2\text{O}$ , 40 cm.  $\text{H}_2\text{O}$ , 75 cm.  $\text{H}_2\text{O}$  and 110 cm.  $\text{H}_2\text{O}$ . Blank experiments were first done with these various pressures at  $0^\circ \text{C}$ . and  $15^\circ \text{C}$ . on 0.2 *M* phosphate buffer pH 7.6 containing 0.1 *M* cyanide. These were used in allowing for the amount of  $\text{CO}_2$  taken up in physical solution during the rapid phase, assuming that the solubility of  $\text{CO}_2$  in the phosphate buffer is the same as in blood. This assumption is almost certainly right to within 10 p.c., and since the total  $\text{CO}_2$  taken up in the rapid phase was usually three to four times greater than that taken up in physical solution, the error from this source should not exceed 3 p.c. The mode of calculation may be illustrated by an example on reduced blood at  $0^\circ \text{C}$ .:

For the reduced blood, initial pressure of $\text{CO}_2$ at $t=0$ was	...	...	110.2 cm. $\text{H}_2\text{O}$
Extrapolation of the slow phase of the curve back to $t=0$ meets the pressure axis at (as in Fig. 5)	...	...	94.2 cm. $\text{H}_2\text{O}$
Therefore total $\text{CO}_2$ taken up rapidly at pressure of $\text{CO}_2$ is equivalent to	...	...	16 cm. $\text{H}_2\text{O}$
For the phosphate buffer initial pressure of $\text{CO}_2$ at $t=0$ was	...	...	110.9 cm. $\text{H}_2\text{O}$
Extrapolation of slow phase back to $t=0$ meets pressure axis at	...	...	104.5 cm. $\text{H}_2\text{O}$
Therefore $\text{CO}_2$ taken up in physical solution at pressure of $\text{CO}_2 = 104.5$ cm. $\text{H}_2\text{O}$ is equivalent to	...	...	6.4 cm. $\text{H}_2\text{O}$

So  $\text{CO}_2$  taken up in physical solution at pressure of  $\text{CO}_2 = 94.2$  cm.  $\text{H}_2\text{O}$   
 would be equivalent to ... .. 5.8 cm.  $\text{H}_2\text{O}$   
 Therefore  $\text{CO}_2$  taken up in rapid chemical combination by the blood is  
 equivalent to 16-5.8 ... .. = 10.2 cm.  $\text{H}_2\text{O}$   
 With the aid of the calibration factor for the boat this gives an answer  
 of 15.7 vols. p.c.  $\text{CO}_2$  for pressure of  $\text{CO}_2$  ... .. = 94.2 cm.  $\text{H}_2\text{O}$   
 Or, allowing for HCN error, 16.0 vols. p.c.  $\text{CO}_2$  for pressure of  $\text{CO}_2$  ... = 69 mm. Hg.

The main lines of our argument and procedure may, for clearness, be recapitulated as follows (*vide* Fig. 5):

- A. The  $\text{CO}_2$  taken up rapidly by normal blood is the sum of
  - (i) that taken up in physical solution;
  - (ii) that taken up in some non-bicarbonate form;
  - (iii) that taken up as bicarbonate.
- B. The  $\text{CO}_2$  taken up rapidly by cyanide blood is the sum of
  - (i) that taken up in physical solution;
  - (ii) that taken up in non-bicarbonate form.
- C. The  $\text{CO}_2$  taken up rapidly by a phosphate buffer is simply that taken up in physical solution.

Therefore C alone gives the physically dissolved  $\text{CO}_2$ ;

B-C gives the non-bicarbonate  $\text{CO}_2$ ;

A-B gives the bicarbonate  $\text{CO}_2$ ;

and thus, by poisoning the carbonic anhydrase, it is possible to sort out from one another two separate reactions of  $\text{CO}_2$ , which in normal blood are indistinguishable by this technique, since in the normal case both reactions proceed rapidly.

*“Non-bicarbonate”  $\text{CO}_2$  dissociation curves of oxygenated and reduced blood.*

In this manner the amount of rapidly bound non-bicarbonate  $\text{CO}_2$  in volumes p.c. was determined at three or four different pressures of  $\text{CO}_2$  both for oxygenated and for reduced blood at  $0^\circ\text{C}$ . and at  $15^\circ\text{C}$ . The results are given in Table I and are also plotted in Fig. 6. The curve relating “non-bicarbonate”  $\text{CO}_2$  content to pressure of  $\text{CO}_2$  may be called the “non-bicarbonate  $\text{CO}_2$  dissociation curve,” and is assumed to be practically the same for normal blood and for cyanide blood.

On inspection of Fig. 6 two things immediately stand out: First, reduced blood has a greater “affinity” for non-bicarbonate  $\text{CO}_2$  than oxygenated blood has: at any given pressure of  $\text{CO}_2$  there is 3 vols. p.c.

TABLE I.

Condition of blood	Temp.	P <sub>CO<sub>2</sub></sub> in mm. Hg	Vols. p.c. in non-bicarbonate CO <sub>2</sub>
Reduced	0° C.	10.6	9.8
	0° C.	22.0	11.6
	0° C.	50.0	14.0
	0° C.	69.0	16.0
Oxygenated	0° C.	11.6	40.0
	0° C.	20.4	9.1
	0° C.	63.2	11.9
Reduced	15° C.	12.0	3.4
	15° C.	23.6	5.7
	15° C.	42.5	7.2
	15° C.	65.0	9.5
Oxygenated	15° C.	12.3	1.2
	15° C.	35.4	4.9
	15° C.	65.0	4.9

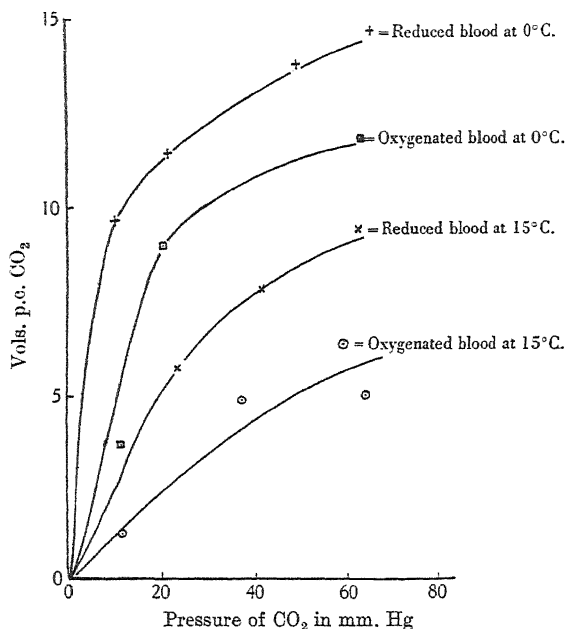


Fig. 6. Non-bicarbonate dissociation curves of oxygenated and reduced blood at 0° C. and 15° C.

or more difference between the two curves. Secondly, the "affinity" of blood for non-bicarbonate CO<sub>2</sub> is much reduced by rise of temperature from 0° C. to 15° C. It may also be noted that the largest amount of non-bicarbonate formation, viz. 15–16 vols. p.c. CO<sub>2</sub>, is not much different from the O<sub>2</sub> capacity of blood, and is certainly appreciable in comparison



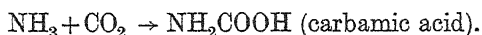
with the total amount of  $\text{CO}_2$  carried by the blood. Further discussion of the chemical and physiological significance of the results must be reserved until the next two sections.

### SECTION III. CARBAMATE COMPOUNDS.

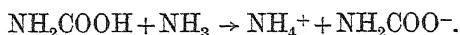
Henriques [1928] has brought forward recently the possibility that  $\text{CO}_2$  combines directly with the blood proteins to form components of a carbamate type: it has also seemed to us very probable that the rapidly formed  $\text{CO}_2$  compound studied in the last section is a hæmoglobin carbamate. In this section we give strong evidence in favour of this view, but before doing so it will be well to recapitulate briefly the relevant properties of carbamate compounds, in so far as these have been studied in simpler cases than the proteins. The chemistry of carbamates has been investigated very fully by Siegfried and their physical chemistry by Faurholt [1925]. It is largely to these authors that our present knowledge is due:

(i)  $\text{CO}_2$  combines very rapidly with ammonia, amines such as amino acids, alcohols, etc., to form carbamate compounds.

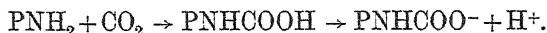
In the case of ammonia the equation is



Carbamic acid being a relatively strong acid reacts with further ammonia to form ammonium carbamate thus:



In the general case of an amine  $\text{PNH}_2$  we have



Faurholt has shown that it is only free  $\text{CO}_2$  (and not  $\text{H}_2\text{CO}_3$ ,  $\text{HCO}_3^-$  or  $\text{CO}_3^{=}$ ) that can react with the amine to form the carbamate.

(ii) The heat of formation of carbamates is very high, *i.e.* of the order of 20,000 calories or more per mol  $\text{CO}_2$ : in agreement with this is the fact that they are most readily formed at  $0^\circ \text{C}$ ., and are largely decomposed by heating to  $70^\circ \text{C}$ .

(iii) The calcium and barium salts of the carbamates are quite soluble in water. This gives a ready method of separating carbamates from carbonates.

(iv) The effect of *pH* upon the equilibrium between  $\text{CO}_2$  and several amines, *i.e.* ammonia, glycine, methylamine, etc., has been carefully studied by Faurholt [1925]. It will be noted from his curves that the

percentage of chemically bound  $\text{CO}_2$  in the form of carbamate increases very markedly as the  $p\text{H}$  goes from 8 to 10, and only declines beyond that because the amount of free  $\text{CO}_2$  in solution available to take part in the equilibrium  $\text{PNH}_2 + \text{CO}_2 \rightleftharpoons \text{PNHCOOH}$  becomes so minute.

In regard to (iv) we have confirmed Faurholt's conclusions by means of the boat technique. Five solutions of  $p\text{H}$  ranging from  $p\text{H}$  8 to  $p\text{H}$  10, containing in one series 0.2  $M$  ammonia and in the second series 0.2  $M$  glycine, were successively shaken with a standard pressure of  $\text{CO}_2$  at  $0^\circ \text{C}$ .

The rate of uptake of  $\text{CO}_2$  in the two series is plotted in Fig. 7, *A*, *B*. There is an initial rapid uptake of  $\text{CO}_2$  complete in the first 2 min., followed by a long slow uptake.

In these simple instances there is no doubt that the rapid process represents carbamate formation, whereas the slow process represents carbonate and bicarbonate formation.

The relation between amount of carbamate (as given by the initial rapid uptake) and  $p\text{H}$  is shown in Fig. 8, *A*, *B*, where the titration curves of ammonia and glycine are also shown.

The parallelism between the titration curves and the amount of carbamate formation is readily explained on the assumption that  $\text{CO}_2$  can only react with an uncharged amino group. Thus in the case of ammonia  $\text{CO}_2 + \text{NH}_3 \rightarrow$  does occur, but the reaction  $\text{CO}_2 + \text{NH}_4^+ \rightarrow$  does not occur, and in the case of glycine, using the modern Zwitterion theory that the neutral form is  $\text{CH}_2\text{NH}_3^+\text{COO}^-$  and not  $\text{CH}_2\text{NH}_2\text{COOH}$ , the reaction  $\text{CO}_2 + \text{CH}_2\text{NH}_2\text{COO}^- \rightarrow$  does occur, but the reaction  $\text{CO}_2 + \text{CH}_2\text{NH}_3^+\text{COO}^-$  does not occur.

Conversely, the relation between carbamate formation and  $p\text{H}$  in the case of the amino acids does indeed give further evidence in favour of the Zwitterion theory. Striking illustration of this was found by comparing the results for glycine with those found in a further set of experiments, all at  $p\text{H}$  8.2 (borate-phosphate buffer), on 0.2  $M$  solutions of four other amino acids and peptides.

These experiments are summarized in Table II:

TABLE II.

Substance	Alkaline $pK$	p.c. in form $\text{PNH}_2\text{COO}^-$ at $p\text{H}$ 8.2	p.c. carbamate formed
Glycine	10.30	1.0	10
Glycylglycine	8.80	25.0	47
Glycylglycylglycine	8.64	36.5	56
Histidine	9.75	2.8	24
Cysteine	8.70	31.5	62

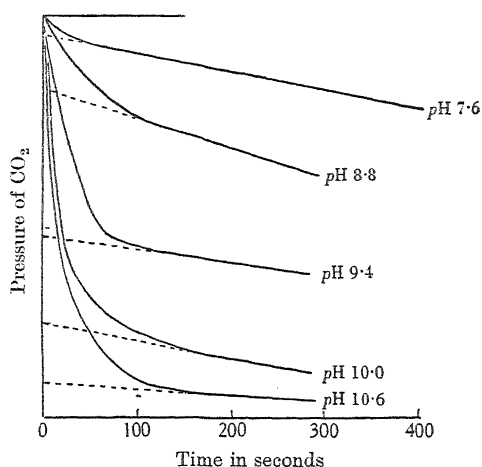


Fig. 7 A.

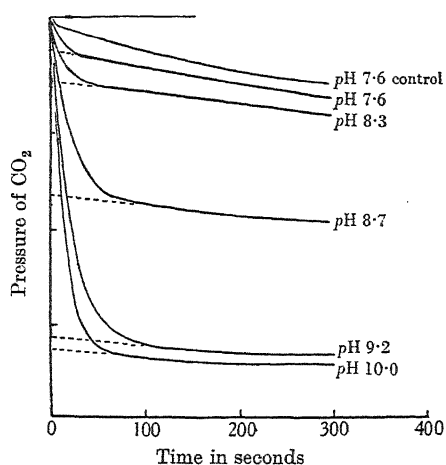


Fig. 7 B.

Fig. 7. A. Rate of uptake of  $\text{CO}_2$  by 0.2M ammonia solutions, pH's as shown. All the curves start from the same point, and the distance between this point and the point, where the flat part of the curve extrapolated back to  $t=0$  meets the pressure axis, represents the amount of carbamate in each case. B. Rate of uptake of  $\text{CO}_2$  by 0.2M glycine at pH's shown. Intercept on pressure axis, by extrapolating flat part of curve back to  $t=0$  gives amount of carbamate as in Fig. 7 A.

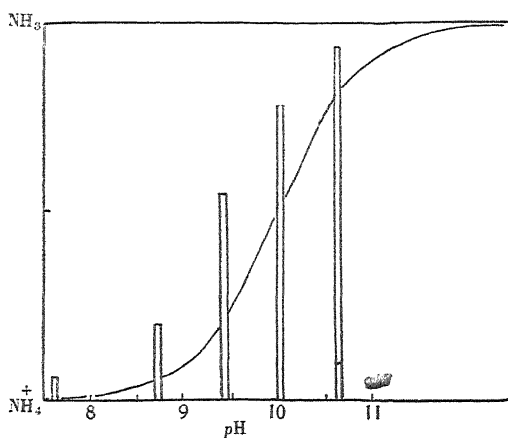


Fig. 8 A.

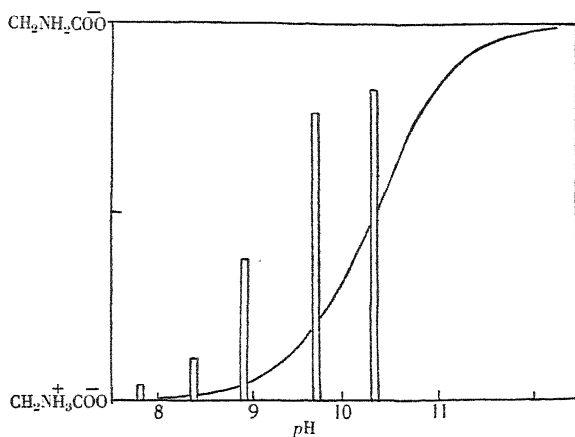


Fig. 8 B.

Fig. 8. A. Smooth curve=titration curve of ammonia. Height of rectangles=proportion of carbamate formed at corresponding pH. B. Smooth curve=titration curve of glycine. Height of rectangles=proportion of carbamino compound formed at corresponding pH.

It is obvious that the larger the proportion of the substance in the uncharged amino form the greater the amount of carbamate formed at given  $pH$ . The parallelism between the effect of peptide formation on  $pK$  and on carbamate formation in the series of glycine and glycylglycine is particularly convincing, and would suggest that a complex protein, if built up by peptide linkages, may well have greater carbamate forming power at a given  $pH$  than have its constituent amino acids.

In the case of proteins on the alkaline side of their isoelectric point, it is accepted that the same type of ionization changes occur as in the simple amino acids and peptides and that these ionizations account for the titration curve and buffer power of the proteins. At physiological  $pH$  the power of a protein to form carbamate should therefore depend on the relative amount in the uncharged  $-NH_2$  form (as compared with that in the  $NH_3^+$ -form), and this will be indicated by the titration curve, or buffer power, of the protein in that  $pH$  range. The steeper the titration curve or the greater the buffer power the greater is the number of ionizations  $NH_3^+ \rightarrow NH_2 + H^+$  functioning, and hence the greater the possibility of carbamate formation. It is well known from the work of van Slyke *et al.*, that hæmoglobin has a steep titration curve and a good buffer power in the physiological range: hence it should have a well-developed power of carbamate formation in that range especially at  $0^\circ C$ . The proteins in plasma only have one-third to one-fourth the buffer power of the hæmoglobin of the blood. It therefore seems natural to conclude that a large part of the rapid  $CO_2$  uptake in the experiments of Section II is due in the main to the formation of carbamino-hæmoglobin compounds. A recent preliminary experiment with ox serum, rendered almost  $CO_2$  free, showed scarcely greater rapid uptake than could be explained by physical solution.

This is further supported by a series of experiments on  $CO_2$  uptake by cyanide poisoned blood at various  $pH$ 's, shown in Fig. 9. The blood was treated with  $0.1M$  KCN + varying amounts of acetic acid and the  $pH$ 's, as calculated approximately from the buffer power of blood and from the buffer power of the  $HCN-CN$  system which acts as a buffer over the range  $pH$  8-10, are appended to the curves. The effect of the  $pH$  upon the amount of  $CO_2$  taken up rapidly in the case of the cyanide blood is thus so very similar to the effect in the case of ammonia and glycine that it is hard to doubt that  $CO_2$  compounds of the same kind are involved in all three cases. Control experiments with a series of similar cyanide solutions in water gave

no greater rapid uptake of  $\text{CO}_2$  than could be accounted for by physical solution.

In preparing  $\text{CO}_2$  free blood for the usual experiments in Section II, it will be remembered that 40 c.c. of blood was treated with 3 c.c. of 0.2*M* acetic acid. If the latter were not added, the *pH* of the  $\text{CO}_2$  free blood would be nearly 1.0 units more alkaline than the usual *pH* of blood, containing 40–50 vols. p.c. bound  $\text{CO}_2$ , and would therefore tend,

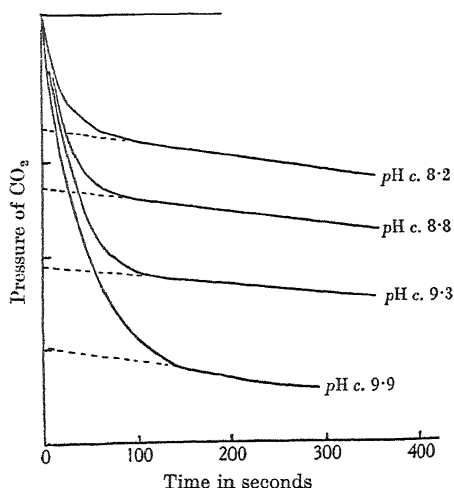


Fig. 9. Uptake of  $\text{CO}_2$  by 0.1 *M* cyanide blood at various *pH*'s. The series of curves show close parallelism with those for ammonia and glycine (*vide* Fig. 8, A, B).

as shown by Fig. 9, to take up more  $\text{CO}_2$  as carbamate than it would do under more physiological conditions. The acid was added to counteract this effect and the amount used was equivalent to roughly two-thirds of the average amount of chemically bound  $\text{CO}_2$  in blood, so that before shaking with  $\text{CO}_2$  began the *pH* of the blood would only be about 0.3 *pH* more alkaline than normal, and after the rapid uptake of  $\text{CO}_2$  is finished the *pH* would probably be quite close to normal, if it is assumed that the carbamino compound so formed is a rather strong acid.

Does the rapidly formed  $\text{CO}_2$  compound in cyanide blood also conform to the properties of the simpler carbamates in regard to items (i), (ii) and (iii) listed at the beginning of this section? We cannot yet

answer this question fully, but our progress so far has been quite encouraging:

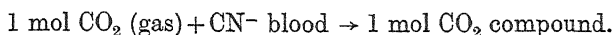
(i) The rate at which  $\text{CO}_2$  combines rapidly with cyanide-poisoned blood seems to be of the same order as in the case of its combination to form the simpler carbamates.

(ii) The heat of formation of the rapid  $\text{CO}_2$  compound in cyanide blood should be roughly calculable from the "non-bicarbonate"  $\text{CO}_2$  dissociation curves at  $0^\circ \text{C}$ . and  $15^\circ \text{C}$ . shown in Fig. 6, by applying the van't Hoff isochore.

If  $p, p'$  are the pressures of  $\text{CO}_2$  required to produce a given rapid uptake of  $\text{CO}_2$  at the two different absolute temperatures  $T, T'$ , then it might be expected that

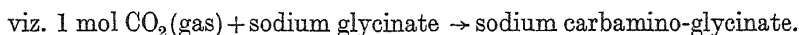
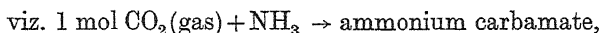
$$\frac{Q}{R} \left( \frac{1}{T} - \frac{1}{T'} \right) = \log p/p',$$

where  $Q$  is the heat of the reaction



This type of equation holds good for the reaction between oxygen hæmoglobin in blood.

The data of Fig. 6 only allow a rough calculation which gives a value of  $Q$  of the order of 20,000 calories. This is quite close to the value of the heat of the reaction,



These two values have been measured approximately by direct calorimetry, and the details will be given elsewhere.

It is hoped, later, to make a direct determination of the heat of the rapid reaction between  $\text{CO}_2$  and cyanide blood.

(iii) Preliminary attempts have been made to separate carbamate compounds in blood by means of a modification of Faurholt's procedure for simpler amines. This consists in making the solution suddenly alkaline with  $\text{NaOH}$  and then mixing with excess of  $\text{BaCl}_2$ . The  $\text{CO}_2$ ,  $\text{H}_2\text{CO}_3$ ,  $\text{HCO}_3^-$  would all be turned very quickly into  $\text{CO}_3^{=}$  which would be precipitated, but not so any preformed carbamate compounds: the latter only dissociate very slowly at alkaline  $p\text{H}$ 's and should therefore be separable from the  $\text{BaCO}_3$  precipitate by centrifuging.

In the case of blood, however, there are a number of difficulties and pitfalls. To overcome these, we have devised a special technique, which seems so far to have succeeded. The results of our first two experiments

have been positive, and indicated that in blood equilibrated with 50 mm.  $\text{CO}_2$  there was a definite amount of  $\text{CO}_2$  which was not precipitated at alkaline  $p\text{H}$ 's by  $\text{BaCl}_2$ , and was therefore presumably in some form other than  $\text{CO}_2$ ,  $\text{H}_2\text{CO}_3$ ,  $\text{HCO}_3^-$  or  $\text{CO}_3^{=}$ . The actual amount agreed rather closely with that to be expected from the "non-bicarbonate"  $\text{CO}_2$  dissociation curves of Fig. 6. It would, however, be undesirable and indeed unwise to go further into this matter, until the method shall have been much more fully tested.

#### SECTION IV. RELATION OF THESE RESULTS TO OTHER WORK ON THE STATE OF $\text{CO}_2$ IN BLOOD, TOGETHER WITH SOME PHYSIOLOGICAL CONSIDERATIONS.

##### *The relation of previous work.*

We shall not discuss any of those pre-1925 papers, which claimed to show that  $\text{CO}_2$  combined directly with the proteins of the blood, since most of that work was gravely handicapped by the fact that the physical chemistry of proteins and of buffers, in particular those present in blood, had not been sufficiently investigated and understood. We shall just summarize and comment briefly on the more up-to-date work, and then consider it in relation to our own.

Three types of method have been used for studying the state of  $\text{CO}_2$  in blood, namely (a) osmotic, (b) electrochemical, and (c) kinetic methods.

##### *(a) Osmotic methods.*

(i) Margaria [1931] found, by A. V. Hill's thermoelectric method, that the vapour pressure of blood was depressed by adding  $\text{CO}_2$  to the blood, but not to the extent expected if the only forms present in the blood were dissolved  $\text{CO}_2$  and bicarbonate. If, however, some of the added  $\text{CO}_2$  combined directly with the blood proteins, or with any other compounds in the blood, such combined  $\text{CO}_2$  would not contribute at all to depression of the vapour pressure, and hence the observed discrepancy would be explained.

(ii) Stadie and O'Brien [1931] carried out similar experiments on the depression of the freezing point of blood. Their results led them to claim, in opposition to Margaria, that the added  $\text{CO}_2$  was osmotically active in the proportion expected from the number of mols added, and hence that no appreciable direct compounds could have been formed. Henriques [1931] has made two criticisms of these results, of which ( $\alpha$ )



on p. 253 of his paper seems to us invalid, but the second one ( $\beta$ ), namely that inconsistent assumptions were made as to the activity corrections, seems to us well founded.

(iii) Henriques [1933] has compared the total osmotic pressure of the interior of the red blood corpuscles with that of the serum, on the assumption that the total osmotic pressure is in each case proportional to the sum of the concentrations of all the solutes present in the two respective phases, the concentrations being measured in mols per litre of water, and the "bound"  $\text{CO}_2$  being reckoned as 100 p.c. osmotically active. Under certain conditions he finds a greater total osmotic pressure in the corpuscles than in the serum, although he contends that there cannot, in view of the properties of the corpuscle membrane, be really any such difference. The discrepancy in the calculated osmotic pressures he attributes to the "bound"  $\text{CO}_2$ , which had been counted as fully osmotically active, but is really only in part osmotically active owing to some of it being in direct combination with proteins. The discrepancy in the total osmotic pressures he takes, indeed, as a measure of the "directly combined"  $\text{CO}_2$ .

In criticism of this ingenious procedure it must be noted that Henriques assumes that the osmotic activity coefficients of the various ions is the same in the corpuscles as in the serum. For this, as far as we know, there is no really satisfactory ground. The seriousness of this objection is shown by the fact that if the osmotic activity coefficient inside the corpuscle was only 5 p.c. less than in the serum, the whole of Henriques' calculated effect would disappear.

The uncertainty of the activity coefficient corrections in strong protein solution is indeed a bar to any rigorous demonstration of direct  $\text{CO}_2$  compounds by osmotic methods, though it cannot be denied that strong measure of probability can be established by such means (as notably in Margaria's experiments).

#### (b) *Electrochemical methods.*

Of these the most important are:

(i) Titration of solutions of sodium hæmoglobinate with  $\text{HCl}$  and with  $\text{CO}_2$  by Hastings, Sendroy, Murray and Heidelberger [1924], and more recently by Stadie and O'Brien [1931]. These authors obtained the same titration curve in the case of the two acids, and hence argued that there was no direct combination of either acid with hæmoglobin, or else that both combined to exactly the same extent, which latter was thought to be a most unlikely coincidence.

Henriques [1931] has, however, criticized the theoretical basis of the calculations in these experiments, and has also pointed out that if  $\text{CO}_2$  combines directly with hæmoglobin to form a carbamino acid, this would be likely, from Faurholt's data, to be an acid with a  $pK$  near 7, and hence would tend to ionize markedly at  $pH$  near 7. This, as he points out, would enable appreciable amounts of such a compound to exist without being detected by the  $\text{CO}_2$  titration curve.

(ii) Donnan equilibrium methods. The partition of bound  $\text{CO}_2$  between the red blood corpuscles and serum or between a hæmoglobin solution and its dialysate, the two being separated by a collodion membrane [Henriques, 1928] fails to agree with the partition of chloride, according to the Donnan equilibrium, if all the bound  $\text{CO}_2$  is in the form of  $\text{HCO}_3^-$ .

The discrepancy can be explained either by a different effect of hæmoglobin on the activity of the  $\text{HCO}_3^-$  ion from its effect on the activity of the  $\text{Cl}^-$  ion, or else by the supposition that some of the bound  $\text{CO}_2$  is present, not as  $\text{HCO}_3^-$ , but in direct combination with the hæmoglobin.

(iii) The Henderson-Hasselbalch equation, viz.

$$pH = pK' + \log \frac{[\text{Bound CO}]}{[\text{H}_2\text{CO}_3]},$$

for the relation between  $pH$ , bound  $\text{CO}_2$  and  $\text{CO}_2$  pressure in blood. In reduced hæmoglobin solutions, Stadie and Hawes [1928] found that the value of  $pK'$  necessary to fit the equations was appreciably lower than in  $\text{CO}$  hæmoglobin solutions at  $15^\circ\text{C}$ . This they attributed to a much larger effect upon the activity coefficient of the reduced hæmoglobin than of  $\text{CO}$  hæmoglobin. The discrepancy can be much more plausibly explained, however, by supposing that a larger amount of the "bound"  $\text{CO}_2$  was combined directly to the reduced hæmoglobin than to the  $\text{CO}$  hæmoglobin.

Margaria [1931, and in unpublished results privately communicated] has tested the Henderson-Hasselbalch equation at very low  $\text{CO}_2$  pressures. He has observed discrepancies, which again can be explained simply by appealing to direct  $\text{CO}_2$  hæmoglobin compounds.

It will be seen that in all these electrochemical methods there is the same difficulty, owing to uncertainty about the effect of strong protein solutions upon the activity coefficients. An opponent of the direct combination theory can always persist in maintaining that hæmoglobin has a specific effect upon the activity coefficient of the bicarbonate ion, and there is no way at present of disproving such a contention directly.

(c) *Kinetic methods*, i.e. that of Henriques, which has been described and discussed in Section I, and the method used in the present paper.

Both these methods depend upon a rather abrupt change at a certain stage in the rate of  $\text{CO}_2$  evolution from, or uptake by, blood. It is very hard to see how the division of the rate curve into two different phases could possibly be due to any "activity" explanation, however far-fetched: we therefore conclude that in this type of experiment we have really unequivocal evidence of direct  $\text{CO}_2$  compounds with the blood proteins. These compounds, for the reasons given in Section III, are almost certainly of a carbamate type.

It remains to compare the results of Henriques with our own. Both have found that a given pressure of  $\text{CO}_2$  reduced hæmoglobin combines directly with more  $\text{CO}_2$  than does oxyhæmoglobin: the difference is of the same order of magnitude in the two researches, when account is taken of the higher p.c. of hæmoglobin in Henriques' solutions. The absolute amounts of directly combined  $\text{CO}_2$  are nearly the same in the range 40–50 mm.  $\text{CO}_2$  pressure, but diverge above and below owing to the fact that Henriques' "non-bicarbonate" dissociation curves are S-shaped, whereas our own are more nearly hyperbolic.

If oxygenation of hæmoglobin affects the direct combination of  $\text{CO}_2$  with hæmoglobin, then conversely the latter should affect the former, i.e.  $\text{CO}_2$  should have a specific effect on the  $\text{O}_2$  dissociation curve of hæmoglobin apart from the change of  $p\text{H}$  which it produces. The effect should be particularly visible at low  $\text{CO}_2$  pressures and at low temperatures. We had intended to verify this directly, but Margaria [private communication] has informed us that he has already done so with success.

In much work on the oxyhæmoglobin dissociation curve, particularly when physiological applications have been held in mind, the blood or hæmoglobin has been equilibrated with gas mixtures containing various pressures of oxygen and  $\text{CO}_2$ . The possible existence of carbamate compounds in such cases complicates the physico-chemical interpretation of the results, and it would, indeed, from this point of view, be better to avoid the presence of  $\text{CO}_2$  altogether. As an instance, the recent paper of Green and Root [1933] may be quoted. These authors obtained a very interesting set of dissociation curves for the blood of certain fishes at various  $p\text{H}$ 's, calculated from the  $\text{CO}_2$  pressure and  $\text{CO}_2$  content of the blood by the Henderson-Hasselbalch equation. It would be satisfactory if their data could be redetermined with  $\text{CO}_2$  free blood, the  $p\text{H}$  being measured by the glass electrode.

*The physiological rôle of carbamate compounds.*

The rôle of carbamates in  $\text{CO}_2$  transport in the blood is probably more important in cold-blooded animals than in warm-blooded, since as shown in Section II they are so much more readily formed in appreciable amounts at low temperatures. This alternative mode of carriage, since it is a rapid one independent of enzymes, may well be of service to animals which have no carbonic anhydrase in their blood.

In order to assess the rôle of carbamates in mammalian  $\text{CO}_2$  transport it would be necessary to have data at  $37^\circ \text{C}$ . Extrapolation from our "non-bicarbonate" curves at  $0^\circ \text{C}$ . or  $15^\circ \text{C}$ . suggests that at  $37^\circ \text{C}$ . and with physiological  $\text{CO}_2$  pressures of 40–60 mm. Hg, reduced blood would probably contain less than 5 vols. p.c., and oxygenated blood less than 2 vols. p.c.  $\text{CO}_2$  in direct  $\text{CO}_2$  combination, and the difference between the two at, say, 50 mm.  $\text{CO}_2$  might be about 3 vols. p.c. These are small figures in comparison with the average  $\text{CO}_2$  content of blood under physiological conditions, viz. 50 vols. p.c., and the supporters of the pure bicarbonate theory may well be nearly correct on a physico-chemical basis as regards  $37^\circ \text{C}$ . though not as regards room temperature and below. From the physiological point of view, it is not only the nature of total  $\text{CO}_2$  in the blood that is important, but also the cause of the difference in the  $\text{CO}_2$  carrying power of arterial and venous blood. Christiansen, Douglas and Haldane [1914] found that, at 50 mm.  $\text{CO}_2$ , reduced blood contained about 5.5 vols. p.c. more  $\text{CO}_2$  than oxygenated blood. If an appreciable fraction of this 5.5 vols. p.c. difference is due to direct  $\text{CO}_2$  protein compounds, then the latter would obviously be physiologically important in  $\text{CO}_2$  transport. Hitherto the difference in the  $\text{CO}_2$  dissociation curve of oxygenated and reduced blood has been attributed to oxyhæmoglobin being a stronger acid than reduced hæmoglobin. Our experiments at  $0^\circ \text{C}$ . and at  $15^\circ \text{C}$ . have, however, shown that a large fraction of the difference in the  $\text{CO}_2$  dissociation curves of oxygenated and reduced blood at  $0^\circ \text{C}$ . may well be due to the direct  $\text{CO}_2$  protein compounds, in view of the marked difference between the non-bicarbonate curves of oxygenated and reduced blood (Fig. 6).

In one case the amount of  $\text{CO}_2$  taken up during the rapid phase by cyanide-poisoned reduced blood was 5 vols. p.c. greater than that taken up during the rapid phase by cyanide-poisoned oxygenated blood, even though the difference in total amounts of  $\text{CO}_2$  taken up finally by the two bloods was probably not more than 7 vols. p.c. We have tried similar experiments at  $37^\circ \text{C}$ ., but these have not yet been quite con-

vincing, for unfortunately the uncatalysed reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$  now goes so fast that it is impossible to separate the rate of  $\text{CO}_2$  uptake into two phases, and this makes the interpretation much more difficult.

The situation, however, is far from hopeless and we hope indeed to settle the question shortly. We are anxious to do so, not only on account of the  $\text{CO}_2$  transport problem, but also on account of the possible effects (*vide* p. 167) of these  $\text{CO}_2$  compounds on the oxygen dissociation curve of blood *in vivo*, and of the changes which may occur in the oxygen dissociation curve after blood is drawn, if that leads to any redistribution of the forms in which  $\text{CO}_2$  is held in the blood. The interest and importance of this matter need no explanation or justification.

#### SUMMARY.

1. Henriques has claimed that when hæmoglobin solutions, containing  $\text{CO}_2$ , are shaken violently *in vacuo*, the  $\text{CO}_2$  comes off in two distinct phases, one very rapid and the other slow and prolonged. This result could only be confirmed for whole blood if the carbonic anhydrase is prevented from acting by poisoning the blood with 0.1M HCN: otherwise the whole of the  $\text{CO}_2$  comes rapidly. It is suggested that Henriques' hæmoglobin solutions contained no effective carbonic anhydrase.

2. For quantitative work the rate of uptake of  $\text{CO}_2$  by  $\text{CO}_2$  free blood is more suitable for study than are  $\text{CO}_2$  output experiments of the Henriques' type.

3. Normal  $\text{CO}_2$  free blood takes up its whole quota of  $\text{CO}_2$  rapidly, when shaken with a gas phase containing  $\text{CO}_2$ . Cyanide-poisoned blood, however, takes up  $\text{CO}_2$  in two phases. Of these the rapid one is due in part to physical solution but in the main to some non-bicarbonate chemical compound, whereas the second slow phase is due to the formation of bicarbonate following on the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$ , which in the present instance proceeds at about its normal rate in absence of catalyst.

4. Controls suggest that 0.1M HCN added to the blood has no effect of importance in the present connection other than the poisoning of carbonic anhydrase.

5. "Non-bicarbonate" dissociation curves are given for the amount of "non-bicarbonate" compound found during the rapid phase at various  $\text{CO}_2$  pressures.

6. Reduced blood forms distinctly more "non-bicarbonate"  $\text{CO}_2$  at a given  $\text{CO}_2$  pressure than does oxygenated blood. Rise of temperature in both cases decreases the amount of the compound greatly, the heat of formation of the non-bicarbonate compound being of the order of 20,000 cal. per mol  $\text{CO}_2$  bound.

7.  $\text{CO}_2$  uptake experiments by ammonia, certain amino acids and peptides, give results so very similar to those with cyanide blood that it may be reasonably assumed that the same type of  $\text{CO}_2$  compound is concerned in all these cases, viz. a carbamino compound. This is confirmed especially by a comparison of the effect of  $p\text{H}$ , and in a preliminary way by the magnitude of the heat of the respective reactions, and the solubility of the barium salts.

8. The place of carbamino compounds in the physical chemistry of  $\text{CO}_2$  transport by blood is discussed in the light of previous work: so also is the question of their importance from a more physiological point of view, especially in regard to the differing  $\text{CO}_2$  carrying powers of oxygenated and reduced blood.

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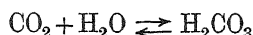
## THE OCCURRENCE OF CARBONIC ANHYDRASE IN LOWER MARINE ANIMALS.

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DURING the work in Cambridge on catalysis of the reaction



Brinkman, Margaria, Meldrum and Roughton (1932) found that although the catalyst is nearly always present in solutions of hæmoglobin, the ratio hæmoglobin/catalytic action is not constant for blood of various animals. In fish blood or frog blood the catalysis for a given amount of hæmoglobin is much less than in mammalian blood, and from the hæmoglobin-containing blood of *Planorbis* and of the earthworm the catalytic action was practically absent. These findings were explained by the discovery of Meldrum and Roughton (1932), who showed that the catalyst could be completely separated from hæmoglobin as an individual and very powerful enzyme, called carbonic anhydrase.

Nevertheless, it remained a striking fact that in the mammalian organism the carbonic anhydrase was found only in fluids or tissues where hæmoglobin was present, *i.e.* in erythrocytes and in red muscles. It might be important to know if this close relation was always maintained or whether one could find instances in which the carbonic anhydrase was present independently of the occurrence of hæmoglobin. In vertebrates the only organ in which the catalyst can be found in cells free from hæmoglobin is in the pancreas; data on this investigation will be given in a later communication.

In lower marine animals, however, the hæmoglobin and the catalyst occur quite independently, and in this paper are communicated some observations, made during a stay at the "Biologische Anstalt" at Heligoland.

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*Methods.*

The enzyme was detected by means of a simple technique, which consisted of estimating the reaction velocity between a 0.006 *N* solution of CO<sub>2</sub> and a 0.02 *N* solution of NaHCO<sub>3</sub> containing phenol red as an indicator. The solutions were separately contained in the stoppered upper branches of a Y-shaped tube, the lower branch being filled with mercury. Mixture of the solutions was effected by inversion of the tube; the reaction took place at a temperature between 0° C. and 1° C., the tube being placed all the time in a beaker with crushed ice. The time between inversion of the tube and attainment of the final yellow colour of the indicator was taken as a measure for the time of reaction, the average time being 75 sec. For testing a (neutralized) solution for presence of carbonic anhydrase a very small amount (0.01 c.c.) was added to the CO<sub>2</sub> solution in the Y-tube. Controls were always taken by adding the same amount or more of the same solution which had been warmed for 2 min. at 80° C.; they should be completely negative, that is the time for change of colour should be at least 75 sec.

The result of this investigation is expressed in the following table:

Species	Fluid examined	Presence of carbonic anhydrase	Remarks
<i>Fucus serratus</i>	Spermatozoa	-	
<i>Rhodomonas</i>	Extract of crushed cells	-	
<i>Aurelia aurita</i> and other types of jelly fish	Body fluid, tissue extracts	-	No Hb
<i>Alcyonium digitatum</i>	Tissue extract	++	"
Actiniæ ( <i>Metridium dianthus</i> )	"	++	"
<i>Asterias</i>	Spermatozoa	+	"
Sea urchin	Body fluid, tissue extract	-	"
"	Eggs	+	"
<i>Aphrodite</i>	Extract of central nervous tissue	-	Hb coloured
"	Body fluid	-	No Hb
"	Extract of muscles	-	"
<i>Nereis pelagicus</i>	Blood	+++	Containing Hb
<i>Amphitrite</i>	"	+++	"
<i>Arenicola</i>	"	+++	"
<i>Lumbricus terr.</i>	"	-	"
<i>Nephtys</i>	Tissue extract	+	No Hb
<i>Ophelia</i>	Blood	-	Containing Hb
<i>Harmotoë</i>	Tissue extract	++	No Hb
Spionidæ	"	+	"
Nemertinae ( <i>Limneus</i> )	"	++	"
<i>Buccinum</i>	Blood	-	Containing Hcy
"	Extract from pharynx muscles	+	Containing Hb
"	Extract from foot muscles	+	No Hb

It is clear from this table that there is, in lower marine animals, no relation between the occurrence of hæmoglobin and of the carbonic



anhydrase. In *Alcyonium* and in Actiniæ for instance the amount of enzyme in tissue extracts is relatively large, although no hæmoglobin can be detected in these organisms. The isolation of the enzyme from Actiniæ (sea anemones, sea roses) was effected on the same lines as it was done from mammalian erythrocytes by Meldrum and Roughton. The dry, slightly yellow powder which resulted after isolation and purification could not be distinguished from the same preparation from ox blood: 1 g., dissolved in 10 c.c. of 1 p.c. NaCl solution, could be diluted 1:10<sup>7</sup>, and still give a definite acceleration of the hydration of CO<sub>2</sub>. 5 min. heating at 60° C. completely destroyed this property.

In worms we find the simultaneous occurrence of hæmoglobin and carbonic anhydrase in *Arenicola*, *Nereis* and other polychætes; in *Lumbricus* and *Ophelia*, however, which contain as large amounts of hæmoglobin, the carbonic anhydrase is absent. Finally in *Harmotoë* the enzyme is distinctly present, but no hæmoglobin can be found. The presence of large amounts of carbonic anhydrase in the blood of *Spirographis* has been shown by H. M. Fox [private communication]. It was already found formerly that *Phascolosoma* (containing hæmerythrin) has a definite amount of the enzyme.

In the snail *Buccinum*, the carbonic enzyme is not present in the blood, but it can be detected in and prepared from extracts of muscles. Now the muscles of the foot are colourless, but some muscles of the pharynx are deeply red from hæmoglobin; the enzyme contents of both types of muscles are quite the same: another proof of the independent occurrence of hæmoglobin and the carbonic anhydrase.

In most of the animals and extracts investigated we have also tested for the presence of catalase and peroxydase (by the guaiacum-H<sub>2</sub>O<sub>2</sub> method). There is no regularity at all in the simultaneous presence of any of the three enzymes: carbonic anhydrase, catalase and peroxydase.

#### SUMMARY.

The occurrence of carbonic anhydrase in a number of lower marine organisms has been investigated. There is no correlation with the distribution either of hæmoglobin, or of catalase or peroxydase.

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## THE GROWTH AND REGRESSION OF FOLLICLES IN THE ŒSTROUS RABBIT.

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### INTRODUCTION.

IN the rabbit ovulation takes place only after copulation, and, in the absence of mating, œstrus may last for months. It has usually been supposed that a single set of mature follicles persists throughout this time. Heape [1905] considered that this was usual, but not invariable, and stated that the formation of blood follicles was the normal result of regression. The absence of these structures in most normal œstrous rabbits has perhaps supported the conclusion, that mature follicles may persist for long periods in the œstrous rabbit; but, in the course of work involving successive laparotomies on the same animals, we were recently led to doubt this view. Specific experiments were then carried out, and, as a result, it appears probable that there is a continuous production and regression of ripe follicles in the unmated rabbit.

Since blood follicles are now known to be an abnormal form of follicular regression, the idea that mature follicles are short-lived in the œstrous rabbit is not incompatible with the comparative scarcity of the hæmorrhagic type.

### TECHNIQUE.

Œstrous rabbits isolated for at least a month previously were chosen for the experiment, and care was taken that all animals were in good condition, as it is well known that follicles may degenerate owing to dietary deficiencies.

In order to trace the life history of the larger follicles, during œstrus, it was necessary to elaborate some method of identifying them at different times and of recording their number and distribution. The idea of attempting a vital staining technique was considered and discarded, because large normal follicles are not readily permeated by vital dyes [Evans, 1916].

It was, therefore, decided to obtain a pictorial record of the ovaries at various intervals during œstrus. Photographic means were first used.

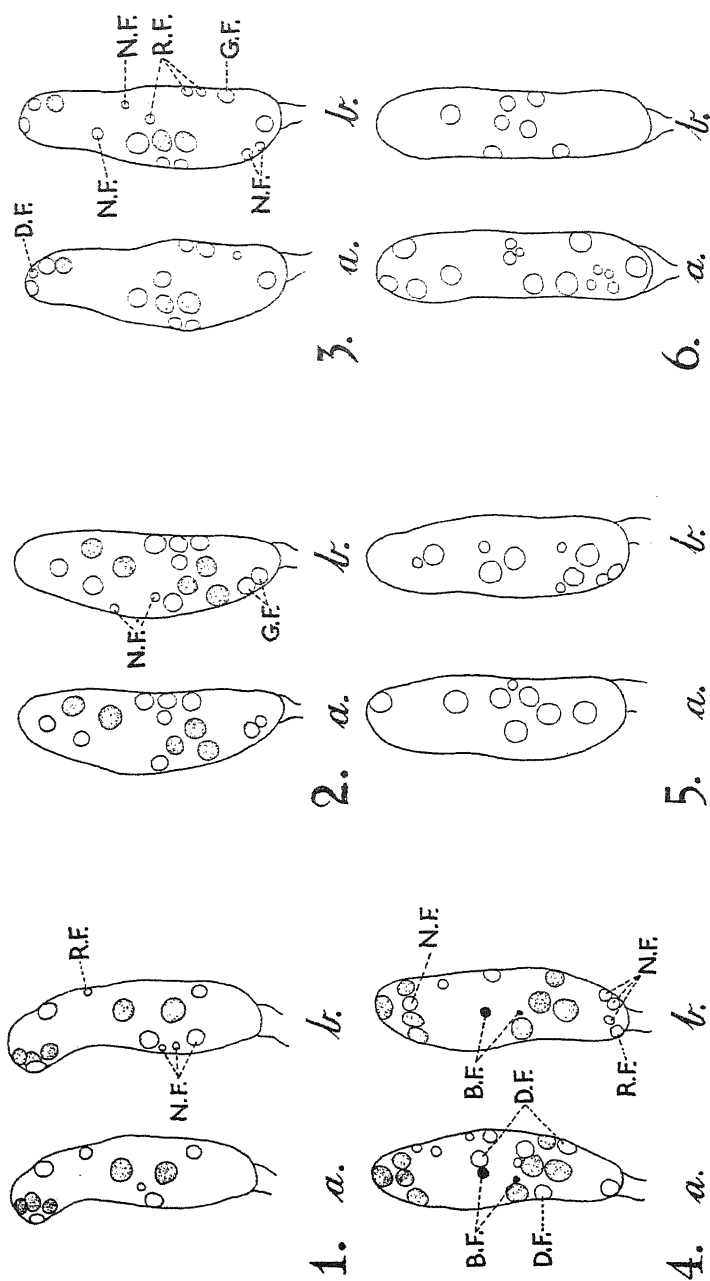
The rabbit was anesthetized with ether and a sterile laparotomy performed. An ovary was then withdrawn, removed from its covering membrane, and orientated so that approximately the same surface area could be photographed each time. Our usual procedure was to place the fimbriæ dorsally. After the photograph had been obtained the ovary was carefully replaced, and the wound closed with catgut.

A Zeiss Greenough binocular camera was used, fixed so that it could be swung directly into position over the ovary, which was never exposed for more than 2-3 min. in all. The lighting, supplied by two 100-watt lamps, was also standardized.

In view of the unexpected results obtained, two control experiments were carried out with the photographic technique. The first consisted in photographing the same ovary twice in 30 min. from a different angle. The result (Pl. I, figs. 4*a* and *b*) shows that slight difference in the orientation of the ovary does not affect the possibility of identifying the follicles in a photograph. The second control experiment was on the effect on the follicles of exposing the ovary to the air. One ovary was photographed through the ovarian bursa, without exposure of the surface to the air, while the other was completely exposed. The subsequent behaviour of the follicles in the two ovaries was not different.

While the manipulation of the ovary for photography did not appear to be a contributory cause in the results observed, it was nevertheless decided in later experiments to reduce the time of exposure by making a rapid drawing of the ovary instead of taking a photograph. All large follicles were indicated on an outline sketch, special attention being given to the size and to the relative position of the follicles to each other and to any old corpora lutea present. This technique proved adequate for the identification of follicles and necessitated exposure of the ovary for a maximum of not more than 1 min.

With present methods it is obviously not possible to ascertain what slight effect, if any, the administration of an anæsthetic may have had.



Text-fig. I.

## RESULTS.

Six Œstrous rabbits were opened and photographs taken of the left and right ovaries of each. PRO 2 and 3 were photographed 27 days later, and PRO 5, 22 days later. In all ovaries the mature follicles appeared to be in different positions as compared with the first examination. It was impossible to recognize any follicle as being one of the original set. It was obvious that the mature follicles present were new and that the original follicles had disappeared completely (see Pl. I, figs. 1*a* and *b*). PRO 4 and 10 were photographed at 14 days after the first examinations. Here again the whole appearance of the ovaries had altered, no follicles being identifiable as belonging to the old group (see Pl. I, figs. 2*a* and *b*). In view of these results, another rabbit (PRO 9) was re-examined after an interval of 7 days, and it was found that the position and number of follicles present in both ovaries were markedly changed even after this comparatively short time (see Pl. I, figs. 3*a* and *b*).

This apparent difference in the mature follicles after only 7 days caused us to repeat the experiments by the more rapid technique of sketching the ovary. Seven ovaries were recorded in this manner at intervals between 7 and 27 days after the first laparotomy. The results were identical with those obtained by the photographic method (see Text-fig. I, 4*a* and *b*, 5*a* and *b*, 6*a* and *b*). After 14 and 27 days the topography of the ovaries had changed completely. After 7 days most of the large follicles had disappeared completely and a number of new follicles had developed. It then seemed necessary to examine the ovaries after shorter intervals to obtain a stage where the large follicles remained unaltered. Five additional ovaries were therefore examined at intervals between 3 and 5 days after the first inspection. The results were as follows:

## Text-fig. I.

Figs. 1*a*-6*a*. Ovaries at first examination.

Figs. 1*b* and 2*b*. Ovaries 3 days after first examination. Growth of one or two new follicles, otherwise ovaries remain unchanged.

Fig. 3*b*. Ovary at 5 days after first examination. Several new follicles grown. One follicle completely disappeared and one or two possibly regressing, but general appearance of follicles unchanged.

Fig. 4*b*. Ovary at 7 days after first examination. Several follicles disappeared completely and new follicles grown; changing appearance of ovary.

Fig. 5*b*. Ovary at 14 days after first examination. Follicles have altered completely.

Fig. 6*b*. Ovary at 27 days after first examination. Similar to 14 days stage.

○ = follicle. ⊗ = corpus luteum. *N.F.* = new follicle. *R.F.* = regressing follicle. *D.F.* = follicle disappeared completely when ovary re-examined. *B.F.* = old blood follicle.

At 3 days, the distribution and number of the mature follicles were unaltered, and except for the presence of one or two small new follicles, the ovaries were comparable in all cases with the initial drawings (see Text-fig. I, 1*a* and *b*, 2*a* and *b*).

At 5 days, the general appearance of the mature follicles was still comparable, but several small new ones had appeared. Of those previously matured, some had decreased slightly in size, while one had disappeared. In addition a follicle previously observed to be small had become mature. The remainder were easily identified as having been present in the same position as at the previous examination (see Text-fig I, 3*a* and *b*).

Since the follicular cycle is not rhythmic in the rabbit, both old and recently mature follicles are probably present at any one examination. If the entire ovary has changed after one week, the survival of mature follicles is probably of the order of 7 to 10 days. This is further indicated by one experiment in which the ovary was completely changed after 7 days, and 5 days later was again different, the largest follicles now being atretic and two or three new follicles having appeared.

#### SUMMARY.

1. Mature follicles do not survive indefinitely in the oestrous rabbit.
2. There is continuous growth and regression of the follicles during the oestrous period.
3. The survival of the mature follicle seems to be from 7 to 10 days so far as can be ascertained by available methods.

The writers wish to express their gratitude to Dr A. S. Parkes for his generous help with the preparation of the manuscript and for criticisms during the course of the experiments.

#### REFERENCES.

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Evans, H. M. (1916). *Proc. Soc. exp. Biol.*, N.Y., 13, 80.

#### EXPLANATION OF PLATE I.

##### *Photographs of Ovaries in situ.*

Figs. 1*a*–3*a*. Ovaries at initial examinations.

Fig. 1*b*. Ovary at 27 days after first examination.

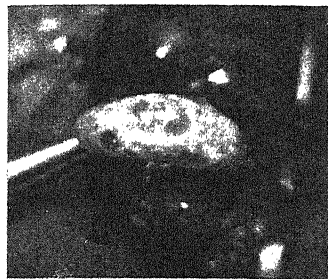
Fig. 2*b*. Ovary at 14 days after first examination.

Fig. 3*b*. Ovary at 7 days after first examination.

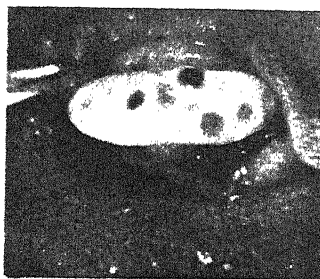
Fig. 4*a*. Control ovary at first examination.

Fig. 4*b*. Same ovary photographed at different angle half-hour after first examination.

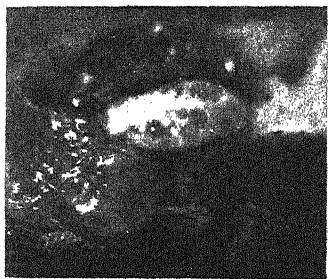
The follicles may be readily identified, although seen in quite a different view.



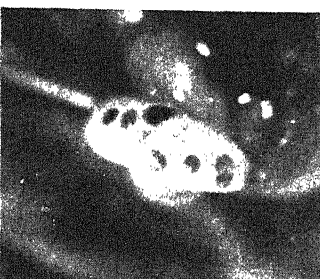
2b



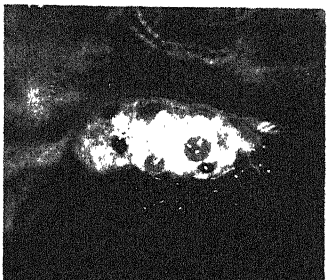
4b



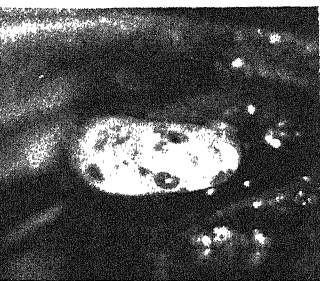
2a



4a



1b



3b



1a



3a





## THE RESPIRATORY QUOTIENT, OXYGEN CONSUMPTION AND GLYCOGEN CONTENT OF THE MAMMALIAN HEART IN AGLYCÆMIA.

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It has been shown that the respiratory quotient of the mammalian heart under hypoglycæmic conditions falls from unity to about 0.75, and this has been taken as indicating that, when the blood sugar is markedly diminished, cardiac muscle falls back on fat and protein in order to meet its energy requirements [Cruickshank and Startup, 1933]. Under the experimental conditions referred to, carbohydrate is not available because of the increase in glycogen synthesis produced by the addition of insulin to the circulating blood. Further, it has been shown that with hypoglycæmia there is a small increase in oxygen consumption and a definite and steady diminution in  $\text{CO}_2$  production.

Further steps in this problem suggested by these results were, to investigate the gaseous metabolism of the heart under a condition of aglycæmia, to determine the lowest level to which the respiratory quotient of the heart muscle would be reduced, and to decide what is the chief source of the energy of contraction and whether or not any transformation of sugar or glycogen from protein and fat may take place.

### METHODS.

The respiratory quotient was determined by a method recently described [Cruickshank and Startup, 1933].

Sugar estimations were made by the Shaffer and Hartmann [1920] method in which the original procedure was modified according to details kindly supplied to us by Prof. Shaffer and since published by Murphy and Young [1932]. In the preparation of the blood-sugar filtrates, the blood proteins and the non-fermentable reducing substances were precipitated by the method of Somogyi [1930]. Lactic acid in blood and

muscle was estimated by a modification of the Fürth-Charnass method described by Lehnartz [1928]. The average percentage recovery by this method has been 99.1, with a maximal error of  $\pm 2.9$  p.c.

Glycogen was estimated by the simplified modification of Pflüger's method described by Sahyun [1931]. This method, whereby glycogen is adsorbed on purified charcoal and hydrolysed in one and the same centrifuge tube, has given excellent results. Cardiac muscle is taken with the least delay possible at the end of the experiment, cut into fairly small fragments, placed in liquid air, and 2-3 g. taken for glycogen estimation.

To make the blood sugar free, the bled dog was given insulin, 5-7 units per kg., about 1 hour before the experiment was performed. Amytal given intraperitoneally was used as the anæsthetic. During the experiment 5 units of insulin were added to the circulating blood every half hour. The attempt to render the heart glycogen free was made by adding 2 c.c. of 1 : 50,000 adrenaline every 15 min. In the experiments quoted adrenaline was added every 30 min.

The total blood volume in the isolated heart-lung preparation was carefully noted and usually was of the order of 600-800 c.c.

## RESULTS.

The results of these experiments were so remarkably uniform that after four experiments with insulin and two with adrenaline aglycæmia we proceeded with another aspect of the problem, and after two months repeated these aglycæmic experiments in order to see if this striking agreement would still obtain. It did so. The protocol published is that of the last experiment on insulin aglycæmia.

### *The glycogen content of the mammalian heart.*

To determine the approximate correctness of our previous assumption that the glycogen content of the experimental heart is not less than 0.500 g./100 g. we carried out several experiments in which the heart was perfused with blood the original sugar content of which was not less than 0.100 g./100 c.c. Insulin was added and the amount of blood sugar disappearing and the oxygen consumption carefully noted. The sugar loss in excess of oxygen utilized was regarded as sugar stored by the heart muscle; this was subtracted from the glycogen content and the original figure thus estimated.

Table I shows by this method an average figure for the original glycogen content of 0.582 g./100 g. In section G of this table is given the

TABLE I. Effect of insulin on the glycogen content of the heart in relation to blood-sugar percentage.

Wt. of heart g.	Glycogen found g./100 g.	Blood sugar original g./100 c.c.	Glycogen added g.	Original glycogen g.	Increase p.c.
A. Normal blood sugar: isolated heart.					
50.0	0.797	0.129	0.111	0.574	38.7
76.0	0.778	0.115	0.118	0.624	24.8
60.0	0.714	0.096	0.098	0.550	17.8
Average=0.582 p.c.					
B. Hypoglycæmia: whole animal.					
138	0.480	—			
217	0.565	0.045			
95	0.433	0.020			
111	0.549	0.025			
87	0.640	0.036			
Average=0.533 g./100 g.					
C. Hypoglycæmia: isolated heart.					
Cruickshank and Shrivastava: 5 experiments, average=0.532 g./100 g.					
D. Aglycæmia: isolated heart.					
59	0.554	Nil.			
90	0.560	Nil.			
51	0.652	Nil.			
75	0.515	Nil.			
Average=0.570 g./100 g.					
E. Effect of adrenaline.					
Hypoglycæmia: whole animal.					
126	0.513	0.052			
158	0.656	0.073			
Average=0.584 g./100 g.					
F. Aglycæmia: isolated heart.					
62	0.610	Nil.			
73	0.590	Nil.			
Average=0.600 g./100 g.					
G. Normal heart: glycogen percentage, whole animal.					
	0.636				
	0.513				
	0.551				
	0.650				
Average=0.581 g./100 g.					

Cruickshank and Shrivastava: 7 experiments, average=0.592 g./100 g.

average figure for the heart of the whole animal, namely, 0.581 g./100 g., which is in agreement, both with the previous result and the average, 0.592 g./100 g. These figures while a little higher, probably due to the use of Sahyun's modification, are in remarkable agreement with the average figure of 0.560 g./100 g. given by Visscher and Mulder [1930] for the unworked isolated heart.

Insulin hypoglycæmia (Table IB) would, in the whole animal, appear to be produced by the storage of sugar in the liver and skeletal muscles rather than in the heart, as indicated by an average glycogen content of

0.533 g./100 g. In the isolated heart in which the blood sugar is allowed to fall to a hypoglycæmic level (Table IC) there is no loss of glycogen, the average figure being 0.532 g./100 g. [Cruickshank and Shrivastava, 1930]. In aglycæmia, of 1-2 hours' duration, produced by insulin, there is again no loss of glycogen from the isolated heart muscle (Table ID); in fact there is a slight increase, which may be due to the synthetic action of insulin in the presence of small amounts of sugar in the blood at the commencement of the experiment. It is evident then from a consideration of such figures, the average of which is 0.565 g./100 g., that our previous assumption of the percentage glycogen content of the heart was essentially correct.

The action of adrenaline in so far as its effect on cardiac glycogen is concerned is interesting in that, in hypoglycæmia as in a prolonged aglycæmia, there is no loss of glycogen (Table IE and F).

*The utilization of sugar in aglycæmia.*

In our previous publication [1933] the utilization of sugar by hearts perfused with a maintained blood sugar and having a respiratory quotient of unity was shown to average 5.28 mg. per g. of heart muscle per hour. The figures for sugar utilization in Table II indicate that during the first half-hour period when sugar is still present in the blood and the respiratory quotient is unity the amount of sugar used averages 2.64 mg., that is a utilization at the rate of 5.28 mg. per g. per hour.

In subsequent periods when the respiratory quotient falls below 0.85 there is little or no utilization of sugar. The steady and rapid disappearance of sugar in relation to the fall in the R.Q. is clearly shown by the uniformity of the curves in Fig. 1. It is also evident (Table II and Fig. 1) that a similar response is occasioned by the addition to the blood of 2 c.c. of 1 : 50,000 adrenaline solution every half hour. The rate of sugar utilization during the first 30 min. when the R.Q. is unity is at the average rate of 3.60 mg., which is 7.20 mg. per g. of muscle per hour. At this rate sugar is wholly removed from the blood by the end of the first hour, by which time the R.Q. is below 0.85.

*The oxygen consumption and respiratory quotient of the heart in aglycæmia.*

Table II and Fig. 1 indicate that there is a slight tendency for oxygen consumption to increase as the blood sugar disappears. In the first experimental period with the R.Q. of unity the average oxygen consumption is 4.14 c.c. per g. of heart muscle per hour. It can be seen from average

TABLE II. The effect of insulin and adrenaline on the combustion of oxygen, sugar, protein and fat by the heart in aglycæmia.

Date	Period 30 min.	INSULIN.					R.Q.
		Oxygen c.c./g.	Sugar mg./g.	Protein mg./g.	Fat mg./g.	Calories per g.	
7. ii. 33							
Heart=75.5 g.	1	1.94	2.57	—	—	0.0105	1.11
	2	2.05	1.65	0.77	0.40	0.0136	0.92
	3	2.07	—	—	1.00	0.0094	0.68
	4	2.05	—	—	0.95	0.0088	0.65
	5	2.05	—	—	0.90	0.0083	0.62
14. ii. 33							
Heart=51 g.	1	2.15	2.72	—	—	1.0112	1.01
	2	2.15	2.72	—	—	0.0112	1.00
	3	2.30	1.37	0.79	0.28	0.0115	0.88
	4	2.35	—	1.91	0.26	0.0103	0.78
	5	2.44	—	—	1.19	0.0111	0.69
8. ii. 33							
Heart=90.5 g.	1	1.98	2.33	—	—	0.0096	1.00
	2	1.98	2.33	—	—	0.0096	1.00
	3	2.06	—	0.79	0.64	0.0092	0.74
	4	1.93	—	—	0.92	0.0086	0.70
6. iii. 33							
Heart=84 g.	1	2.15	2.89	—	—	0.0117	0.99
	2	1.99	2.42	0.01	—	0.0105	0.94
	3	2.10	—	0.04	0.85	0.0096	0.72
	4	2.01	—	—	0.98	0.0091	0.69
11. v. 33							
Heart=59 g.	1	2.18	2.94	—	—	0.0118	1.22
	2	2.35	—	0.87	0.75	0.0101	0.74
	3	2.27	—	0.41	0.92	0.0102	0.72
	4	2.38	—	—	1.15	0.0107	0.68
	5	2.35	—	—	1.15	0.0107	0.69
	6	2.27	—	—	1.12	0.0104	0.70
	7	2.13	—	—	1.05	0.0098	0.69
ADRENALINE.							
24. i. 33							
Heart=62 g.	1	3.05	3.90	—	—	0.0167	1.07
	2	3.34	1.42	1.33	0.48	0.0157	0.84
	3	3.60	—	3.34	0.21	0.0156	0.79
	4	4.14	—	0.37	1.79	0.0168	0.71
16. xii. 32							
Heart=84 g.	1	2.48	3.30	—	—	0.0135	1.00
	2	2.66	3.32	—	—	0.0136	0.95
	3	3.15	—	2.51	0.37	0.0137	0.78
	4	3.62	—	0.93	1.14	0.0141	0.73

Average O<sub>2</sub> utilization prepared from Fig. 1.

Insulin: in hypoglycæmia, R.Q. 1.00, 4.14 c.c./g./hour.  
           in hypoglycæmia, R.Q. 0.85, 4.30 c.c./g./hour.  
           in aglycæmia, R.Q. 0.80, 4.36 c.c./g./hour.  
           in aglycæmia, R.Q. 0.70, 4.32 c.c./g./hour.

figures calculated from Fig. 1, that as the R.Q. falls from unity to 0.70 the oxygen consumption increases from 4.14 c.c./g./hour to 4.30 c.c. for an R.Q.=0.85; 4.36 c.c. for an R.Q.=0.80; and finally to 4.32 c.c. for an R.Q.=0.70, a comparatively small change which shows how steady is the oxygen consumption of the heart in the presence of such a marked change

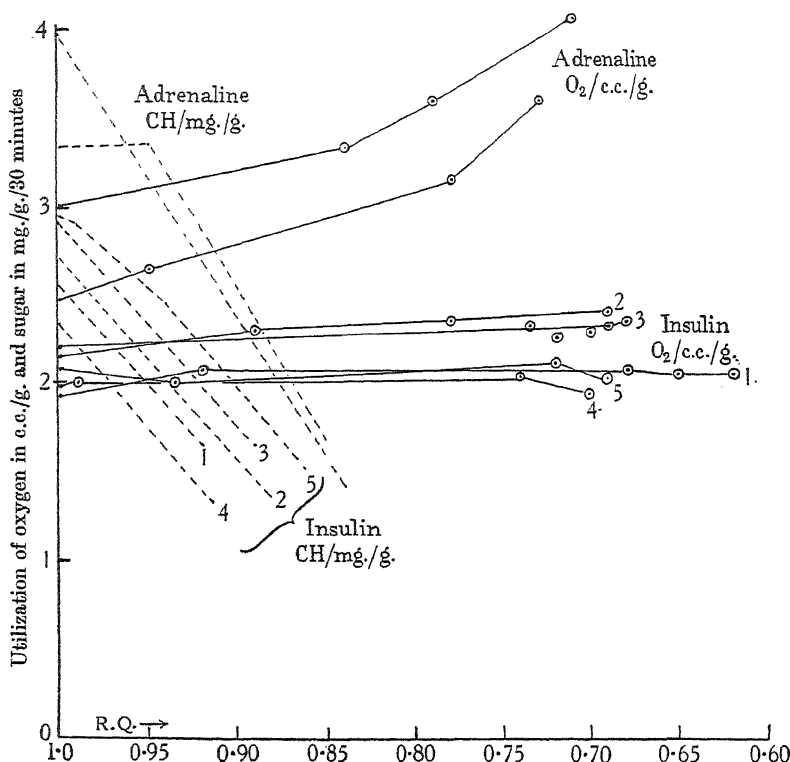


Fig. 1. Curves showing relation between R.Q.'s and the utilization of sugar and oxygen. The time between each point = 30 min.

in the type of foodstuff used for the supply of energy. The slight increase in the amount of oxygen consumed is undoubtedly due to the presence of insulin, as hypoglycæmia, *per se*, produces a slight fall in oxygen utilization.

It is also evident that neither insulin hypoglycæmia nor insulin aglycæmia produces any depression in respiratory metabolism. The addition of adrenaline occasions a marked increase in the utilization of oxygen which is accounted for first by the rapid removal of the blood sugar and secondly by the increased energy needs of the heart.

*The utilization of protein by the heart in aglycæmia.*

In this connection brief reference must be made to the recent revolutionary work in the chemistry of the contraction of skeletal muscle. Lundsgaard [1930-1] has shown that the iodoacetic acid poisoned muscle can contract perfectly well without lactic acid formation and with an almost complete breakdown of phosphagen. That phosphagen is broken down into creatine and inorganic phosphate in muscle activity and may thus supply for a time all the necessary energy for contraction has been demonstrated both by Lundsgaard [1930] and Lipmann and Meyerhof [1930]. Evans [1912] and others many years ago suggested that the isolated heart was able to oxidize the same materials as those used by the body as a whole, but evidence as to the possibility that protein could be burned by the isolated heart is lacking. It would appear as seen from the results in Tables I and II that when cardiac muscle shows an R.Q. of 0.80 in the absence of blood sugar and with no evidence of the utilization of glycogen, protein must of necessity be utilized. The later work of Canzanelli and Rapport [1932], who used the excess R.Q. of exercise as a criterion of the non-nitrogenous metabolism, would indicate that protein, as well as carbohydrate and fat, can be used as fuel in muscular exercise.

A survey of the possible quantities of protein utilized as shown in Table II would tend to the conclusion that there is no great call upon protein, but rather that it forms a very brief transition stage in the change-over from carbohydrate to fat. It may be argued that, since an R.Q. of 0.75 obtains when some 15 p.c. of the total oxygen may be consumed by sugar and 85 p.c. by fat, it is possible that traces of sugar may be present which the methods used do not accurately measure. But in these experiments 0.01 g./100 c.c. of sugar means that there is about 50-70 mg. of sugar available in the circulating blood, and all our experiments show that during the combustion of so small an amount of sugar the R.Q. is never below 0.85. A consideration of the periods shown in Table III indicates that when such a remnant of blood sugar has been finally removed, and if only carbohydrate and fat were utilizing oxygen, then for the R.Q. shown, the amount of sugar burned or the amount of oxygen that has been used for the combustion of the remaining sugar is in no way sufficient to meet the percentages for the distribution of the oxygen burned at the R.Q. obtaining [Zuntz and Schumburg, 1901].

For example, in the first period quoted, 70 mg. of sugar are finally burned; this demands 52.50 c.c. of oxygen. The R.Q. for the period is 0.88,

and at such an R.Q. if only carbohydrate and fat are oxidized the amount of the oxygen consumed by sugar would be 59 p.c. of the total, that is, in this period it would be 66.91 c.c. When the blood is absolutely sugar free at the end of such a period and the R.Q. is still as high as 0.88 the fact that protein may here be utilized cannot be ignored. Further, when 52.50 c.c. have been removed from the total amounts of  $\text{CO}_2$  and  $\text{O}_2$ , the

TABLE III. Periods showing evidence of combined oxidation of sugar, protein and fat.

	INSULIN.			Sugar used mg.	$\text{O}_2$ equivalent sugar used c.c.
	$\text{CO}_2$ c.c.	$\text{O}_2$ c.c.	R.Q.		
Exp. 14. ii. 33					
Period No. 3.	104.34	118.49	0.88	70	52.50
Subtracting	52.50	52.50	1.00		
we have	51.84	65.99	0.76		
With R.Q. = 0.76					
Protein = 60 p.c.	= 31.10	38.77			
Fat = 40 p.c.	= 20.74	29.62			
	51.84	68.39			
				$\text{O}_2$ balance = -2.40 c.c.	
				S.	P. F.
				50.3	29.8 19.9
Period No. 4.	94.16	120.21	0.78		
With R.Q. = 0.78					
Protein = 80 p.c.	= 75.33	94.16			
Fat = 20 p.c.	= 18.83	26.90			
	94.16	121.06			
				$\text{O}_2$ balance = -0.85 c.c.	
				S.	P. F.
				0	80 20
Period No. 5.	86.53	124.84	0.69		
With R.Q. = 0.69					
Fat = 100 p.c.	= 86.53	123.61			
				$\text{O}_2$ balance = 1.23 p.c.	
				S.	P. F.
				0	0 100
Exp. 7. ii. 33.					
Period No. 2.	142.52	154.88	0.92	126	94.50
Subtracting	94.50	94.50	1.00		
we have	48.02	60.38	0.79		
With R.Q. = 0.79				66.3	32.0 1.7
Protein = 95 p.c.	= 45.62	57.02			
Fat = 5 p.c.	= 2.40	6.29			
	48.02	63.31			
				$\text{O}_2$ balance = -2.93 c.c.	
				S.	P. F.
				0	0 100
Period No. 3.	106.89	156.97	0.68		
With R.Q. = 0.68					
Fat = 100 p.c.	= 106.89	152.70			
				$\text{O}_2$ balance = 4.27 c.c.	



TABLE III (*cont.*).

ADRENALINE.					
	CO <sub>2</sub> c.c.	O <sub>2</sub> c.c.	R.Q.	Sugar used mg.	O <sub>2</sub> equivalent sugar used c.c.
Exp. 24. i. 33.					
<i>Period No. 2.</i>	173.06	207.00	0.84	88	66.00
Subtracting	66.00	66.00	1.00		
we have	107.06	141.00	0.76		
With R.Q. = 0.76					
Protein = 60 p.c.	= 64.23	80.29			
Fat = 40 p.c.	= 42.83	61.19			
	107.06	141.48			
				O <sub>2</sub> balance = - 0.38 c.c.	
				S. P. F.	
				31.8 43.6 24.6	
<i>Period No. 3.</i>	178.15	223.56	0.79		
With R.Q. = 0.79					
Protein = 90 p.c.	= 160.33	200.41			
Fat = 10 p.c.	= 17.82	25.46			
	178.15	225.87			
				O <sub>2</sub> balance = - 2.31 c.c.	
				S. P. F.	
				0 90 10	
<i>Period No. 4.</i>	183.24	257.60	0.71		
With R.Q. = 0.71					
Protein = 10 p.c.	= 18.32	22.90			
Fat = 90 p.c.	= 164.92	235.60			
	183.24	258.50			
				O <sub>2</sub> balance = - 0.90 c.c.	
				S. P. F.	
				0 0 100	

non-carbohydrate R.Q. is in this case 0.76, which indicates that the remaining oxygen has been utilized by protein and fat for their combustion in the ratio of 60 to 40 p.c. Similar results are shown in Table III for the experiment dated 7. ii. 33.

In the following half-hour period the heart deprived of all blood sugar utilizes protein and fat in the ratio of 80 to 20 p.c. From a previous consideration of the glycogen content of these aglycæmic hearts under the influence of insulin, where it has been shown that little or no change takes place in the carbohydrate reserve of the heart, and in view of the fact that, coupled with the evidence obtained from the R.Q.'s there is a slight increase in the total nitrogen of the blood, we are further investigating the possibility that cardiac muscle in its transition from the utilization of sugar to that of fat may make use of some of the more easily oxidizable amino acids.

*The possibility of the formation of glycogen from protein.*

While it is argued from the results obtained here, where an R.Q. in the neighbourhood of 0.80 is maintained for at least 30 min., that protein may possibly be utilized, it cannot be demonstrated by R.Q.'s alone without further enquiry into the nitrogenous metabolism of the heart muscle. That the normal animal body can form glycogen from protein has been demonstrated by many [Atkinson, Rapport and Lusk, 1922]. The nature of the conversion depends upon the type of deamination of the amino acids, but the important point here is whether or not the conversion depends upon the presence of the liver. Mann and Magath [1922-3] maintain that the conversion is entirely a function of the liver, but results based on experiments on dehepatized animals may be misleading, and the possibility that deamination is a function of tissue in general is not yet overthrown. It is, however, impossible to arrive at any conclusion from the results here noted. All that can be said is that the rapid fall of the R.Q. to, and its retention at, 0.70 with the absence of any change in the glycogen content of the heart would indicate that while protein and carbohydrate derived therefrom may be used for fuel as suggested for the whole organism by Macleod [1928] such an event must be of a transient nature.

*The changes in lactic acid in blood and heart muscle.*

The lactic acid content of the heart muscle appears to vary slightly with the degree of reduction of blood sugar. The average of five normal hearts is 82.67 mg./100 g. The hearts from six hypoglycæmic bleeders gave an average of 40.40 mg./100 g., while the average for five aglycæmic experimental hearts was 41.49 mg./100 g. The average for the lactic acid content of whole blood estimated by West's method is 37.6 mg./100 c.c. for defibrinated blood; in mild hypoglycæmia it is 33.0 mg./100 c.c. In marked hypoglycæmia, the condition existing at the commencement of these experiments, where the blood sugar is not above 0.035 g./100 c.c., it is 17.8 mg./100 c.c., at the end of the first and second hour of aglycæmia the average percentage amounts are 16.30 and 10.76 respectively. In these experiments the estimation of lactic acid in aglycæmic and markedly hypoglycæmic blood was carried out by the method of Lehnartz [1928]. These figures are sufficient to show that changes in lactic acid during the course of an experiment may be regarded as negligible as far as any change in oxidative processes may be concerned.

*The utilization of fat by the heart in aglycæmia.*

Cardiac muscle like skeletal muscle does perform work by oxidizing preformed carbohydrate, but, as carbohydrate disappears from the circulating blood, as in hypoglycæmia *per se* or by the injection of insulin, there is definite evidence that carbohydrate no longer forms the sole fuel of activity [Cruickshank and Startup, 1933]. The question then arises, what is the final source of energy in the heart supplied with sugar-free blood? Is it preformed carbohydrate, protein, or fat? That the preformed carbohydrate supplies of the heart are not utilized has already been noted, and the possibilities with regard to protein utilization have been discussed. We are left then to discuss the evidence concerning fat combustion.

A considerable difference of opinion has arisen on the question of whether or not mammalian tissue can directly oxidize fat. Leathes [1906] and Winfield [1915] could find no evidence for the consumption of fat by normal muscle. Palazzolo [1912] found a great diminution in the fat content of stimulated muscles of both the frog and the hedgehog. Such experiments do not settle the question of direct oxidation, nor do they form evidence that the liver is the sole site for the desaturation of fats. The only evidence for direct oxidation of fat by muscle tissue comes from Lafon [1906]; it is a piece of evidence which, it would appear, has never been confirmed nor contradicted.

Few observations exist on the R.Q. of isolated skeletal muscle. Meyerhof and Himwich [1924] concluded, from experiments on the diaphragm of the rat, that the R.Q.'s were not dependent on previous diet, and, since they averaged 0.95, that sugar was the chief source of energy. Takane [1926], using a similar preparation, concluded that fat as well as protein and carbohydrate were oxidized by the muscle. We have already shown [1933] that the oxidative metabolism of the heart is exclusively carbohydrate in the presence of available sugar. This is in agreement with the results of the numerous investigations of Meyerhof, Hill and their collaborators on isolated muscle, and of Bornstein [1929] and Corkill, Dale and Marks [1930] for the eviscerated preparation. Numerous experiments upon the R.Q. of resting [Himwich and Castle, 1927] and on the contracting muscle [Himwich and Rose, 1929] would indicate that the R.Q. of normal muscle is that of the whole body, which would imply the oxidation within the muscle of a considerable amount of fat, whether directly or indirectly is an open question. That fat is utilized in exercise has been unquestionably demonstrated by

TABLE IV. Tabular protocol of one experiment.

Date: 11. v. 33.				Bar. = 761 mm. Hg.			
Wt. of dog = 11.5 kg.				Temp. = 20.75° C.			
Wt. of heart = 59.0 g.				Factor = 0.912.			
Heart glycogen = 0.544 g./100 g.							
0.554 g./100 g.							
Period 30 min.	CO <sub>2</sub> c.c.	O <sub>2</sub> c.c.	R.Q.	Heart rate beats per min.	Alveolar blood- pressure mm. Hg.	Blood in re- servoir c.c.	Remarks
1	147.61	130.05	1.140	150	85	460	
2	101.80	138.43	0.735	160	90	450	Insulin 5 units 10 c.c. blood taken
3	96.71	134.25	0.720	162	90	440	Insulin 5 units 10 c.c. blood taken
4	96.71	140.53	0.688	170	98	440	
5	96.71	138.43	0.690	160	96	435	Insulin 5 units
6	94.16	134.25	0.701	164	94	435	
7	88.08	125.86	0.698	162	92	430	Insulin 5 units
<i>First Period.</i> R.Q. = 1.140.							
Blood sugar: begin. = 0.026 g./100 c.c.; end = 0.006 g./100 c.c.							
Blood sugar used = 0.170 g. O <sub>2</sub> = 130.05 c.c. ≡ 0.174 g. sugar.							
	CO <sub>2</sub> c.c.	O <sub>2</sub> c.c.					
	147.61	130.05					
<i>Second Period.</i> R.Q. = 0.735.							
Blood sugar: begin. = 0.006 g./100 c.c.; end = nil.							
	101.80	138.43					
From CO <sub>2</sub> :	Protein = 35 p.c. =	35.63	R.Q. = 0.8 =	44.54			
Fat	= 65 p.c. =	66.17	R.Q. = 0.7 =	94.53			
	101.80	139.07	O <sub>2</sub> = - 6.64 c.c.				
<i>Third Period.</i> R.Q. = 0.720.							
	96.71	134.25					
From CO <sub>2</sub> :	Protein = 20 p.c. =	19.34	R.Q. = 0.8 =	24.7			
Fat	= 80 p.c. =	77.37	R.Q. = 0.7 =	110.53			
		135.23	O <sub>2</sub> = - 0.45 c.c.				
<i>Fourth Period.</i> R.Q. = 0.688.							
	96.71	140.53					
From CO <sub>2</sub> :	Fat = 100 p.c. =	96.71	R.Q. = 0.7 =	138.15	O <sub>2</sub> = + 2.38 c.c.		
<i>Fifth Period.</i> R.Q. = 0.690.							
	96.71	138.43					
From CO <sub>2</sub> :	Fat = 100 p.c. =	96.71	R.Q. = 0.7 =	138.15	O <sub>2</sub> = + 0.28 c.c.		
<i>Sixth Period.</i> R.Q. = 0.701.							
	94.16	134.25					
From CO <sub>2</sub> :	Fat = 100 p.c. =	94.16	R.Q. = 0.7 =	134.90	O <sub>2</sub> = - 0.65 c.c.		
<i>Seventh Period.</i> R.Q. = 0.698.							
	88.08	125.86					
From CO <sub>2</sub> :	Fat = 100 p.c. =	88.08	R.Q. = 0.7 =	125.83	O <sub>2</sub> = + 0.03 c.c.		

Zuntz [1911] and by Anderson and Lusk [1917], the latter workers obtaining R.Q.'s in the fasting dog of 0.71-0.73. The generally accepted conclusion from the work of Hill, Long and Lupton [1924] is that in mild exercise carbohydrate is used, but, that as the exercise is increased or becomes prolonged or more violent fat is drawn upon to supply the needed sugar, with a resultant fall in the R.Q. On the other hand Rapport and Ralli [1928], from the results of similar experiments on dogs, concluded that even in mild exercise the animal oxidized practically the same percentage of fat and carbohydrate as at rest, and that muscles, like other tissues in the body, oxidized carbohydrate or fat, or both, depending upon the proportions in which these substances were presented to them in available form [Rapport, 1929].

It is clear, from the evidence derived from R.Q.'s, that normal muscle can and does utilize fat. There is no doubt that this fact holds good for cardiac muscle. When a heart maintains a rate of 160 beats per min., a blood-pressure of 90-100 mm. Hg for 3 hours with not a trace of blood sugar, and an R.Q. varying between 0.72 and 0.68 with an average over five 30-min. periods of 0.699 (Table IV), then there is no question of the ability of the isolated heart muscle to utilize fat. Such results as are shown in Tables II, III and IV clearly demonstrate this fact, as well as the fact that cardiac muscle in the absence of the liver can, by desaturation, prepare the fat for oxidation. Whether it is an exclusive oxidation of fat or a transformation of fat to carbohydrate cannot be determined by the R.Q.'s alone. A further discussion of this question will form part of a subsequent paper on the changes in the R.Q. of the diabetic heart.

#### SUMMARY.

The glycogen content of the mammalian heart under a condition of strict aglycæmia has been determined, and it has been shown that little or no change is effected in the carbohydrate stores in the presence of insulin or adrenaline aglycæmia.

In the production of aglycæmia the heart muscle utilizes available blood sugar at the rate of 5.28 mg. per g. of heart muscle per hour.

The oxygen consumption of the heart in aglycæmia varies from 4.14 to 4.36 c.c. per g. of muscle per hour, showing that with a fall in the R.Q. from unity to 0.70 there is but little change from the normal oxygen utilization.

With a rapid disappearance of blood sugar the R.Q. falls within 30-60 min. to 0.70, the rate of fall depending upon the amount of sugar left in the blood at the commencement of the experiment.

The R.Q. of cardiac muscle falls from unity when the blood-sugar percentage is less than 0.03 g./100 c.c. There is evidence from the R.Q. that for a short period during and after the disappearance of blood sugar, protein is being utilized, and that such a period is a brief transitional stage from the utilization of carbohydrate to that of fat.

An R.Q. of 0.70 maintained for two or more hours under aglycæmic conditions is accepted as evidence that the heart muscle resorts quickly and finally to fat as the source of its energy.

It has been demonstrated that neither insulin nor adrenaline will effect a diminution, far less a depletion, of the glycogen content of the mammalian heart.

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## RELATION OF THE PITUITARY GLAND TO THE ACTION OF INSULIN AND ADRENALINE.

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### INTRODUCTION.

THAT the pituitary gland plays an important rôle in carbohydrate metabolism has been suspected for a number of years. In 1911 Cushing observed, both clinically and experimentally, that pituitary deficiency was accompanied by an increased sugar tolerance, and references which he quotes indicate that such a relation had been suggested at an even earlier date. Very little further progress was made until 1925, when Houssay and his collaborators embarked upon detailed investigations, which have thrown much light on the problem and have led to some striking results. It is not possible here to review the large number of papers which have appeared both in the *Compte rendu de la Société de Biologie*, and in the *Revista de la Sociedad Argentina de Biología* under the name of Houssay and his associates, but the results have been very well summarized in several papers which are more easily accessible [Houssay, 1931, 1932].

In short, Houssay has come to the following conclusions:

(1) That the pituitary gland is mainly responsible for the new formation of sugar from non-carbohydrate sources—in particular from endogenous protein, and possibly also from fat.

(2) In phlorrhizin and pancreatic diabetes, where gluconeogenesis is thought to proceed at a greatly increased rate, removal of the pituitary gland leads to the suppression of this excessive new formation of sugar, with consequent reduction of the glycosuria and conservation of body protein. So striking is this effect that, in the case of the totally depan-

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creatinized dog, practically all the symptoms of diabetes disappear after removal of the pituitary [Houssay and Biasotti, 1930].

(3) The diminution in endogenous protein metabolism under the above conditions is indicated by a fall in creatinine elimination, and in fat metabolism, by diminished production of acetone bodies.

(4) Converse changes are brought about by excessive pituitary action. Thus repeated injection of a suitable extract of anterior lobe produces a condition of hyperglycæmia and acidosis very similar to pancreatic diabetes.

In this connection, Houssay has presented a good deal of evidence to show that the anterior lobe is the part of the gland immediately concerned in these effects, while Geiling and his colleagues consider the posterior lobe to be responsible. Cushing first thought that the posterior lobe was responsible, but later attributed the effects to the anterior lobe.

(5) In harmony with the observations just cited on the suppression of pancreatic and phlorrhizin diabetes, removal of the pituitary body leads also to a hypersensitiveness to insulin. Thus a small dose of insulin, which in the normal animal would entail only a transient hypoglycæmia, produces in the absence of the pituitary body a progressive fall in blood sugar to a convulsive level with sometimes complete failure to rise again to the normal level. This latter characteristic has been particularly noted by Geiling *et al.* [1927] in dogs.

The explanation of this exaggerated response to insulin on the basis of Houssay's hypothesis would offer little difficulty, if we could assume that the restoration of the blood sugar to the normal level after insulin hypoglycæmia was normally accomplished by an increase in the rate of sugar production from protein and fat through the mediation of the pituitary gland. Removal of this gland, however, is known to produce profound degenerative changes in the thyroid and adrenal glands, and these glands in turn are known to be intimately connected with the maintenance of the normal blood sugar level, through the regulation of the discharge from the liver of glycogen already stored there. It therefore seemed to us worth while to investigate the relation between the increased sensitivity to insulin and the changes in the thyroid and adrenal glands, before proceeding to the more fundamental question of new formation of carbohydrate. The present paper deals mainly with the part played by adrenaline and the adrenal medulla. In future communications it is hoped to extend the investigation to the adrenal cortex and to the thyroid gland.



The evidence concerning the part played by the pituitary body in carbohydrate metabolism has hitherto been derived chiefly from experiments on dogs and a few on monkeys [Hartman, Firor and Geiling, 1930]. Although the rabbit is the species for which by far the most complete data are available as to response to insulin and adrenaline, no experiments have yet been recorded as to the effect on the response of removing the pituitary body. A method of carrying out this operation in the rabbit being now available, it seemed desirable to investigate how far the striking effects produced in the dog are to be observed also in the species from which so much of the detailed knowledge of the mechanism of carbohydrate control has been derived.

#### METHODS.

Rabbits were used in all experiments. Pituitary removal was carried out by one of us [White] by a buccal approach described by Smith and White [1931] and in greater detail by White [1933]. All the animals were finally killed, and the endocrine glands weighed and examined histologically. Pituitary ablation was checked by serial sections of the contents of the sella turcica and in the more critical experiments by serial sections of the whole pituitary region. In most cases samples of liver and muscle were taken for determination of glycogen by the Evans' *et al.* [1931] modification of Pflüger's method. In some cases, also, extracts were prepared from one or both adrenal glands and the adrenaline content determined colorimetrically. We are greatly indebted to Dr H. Schild for kindly undertaking this part of the work.

Determinations of blood sugar were made by the Hagedorn-Jensen method. The course of the blood-sugar curve has been studied following subcutaneous injection of insulin and of adrenaline, and oral administration of glucose. Unless otherwise stated, the hypophysectomized animals were deprived of food for only 12 hours before an experiment, in view of the danger of a fatal hypoglycaemia developing during more prolonged fasting. We have further investigated the effect of pituitary removal upon the deposition of liver glycogen in young rabbits treated with insulin, following an observation originally made by Goldblatt [1929] and later extended by one of us [Corkill, 1930]. Unfortunately young rabbits survive hypophysectomy very poorly. In addition to their general lack of resistance, they have only a thin *diaphragma sellæ* and an imperfectly formed bony capsule, which in the adult almost completely separates the pituitary body from the brain. For these reasons

varying amounts of cerebrospinal fluid generally escape when complete ablation is attempted in very small rabbits. We have found that the loss of any considerable quantity of cerebrospinal fluid is always fatal. Nevertheless, in a few cases the technique has been successful. The number of such experiments is small, but the few results we have obtained are quite definite.

#### THE INFLUENCE OF HYPOPHYSECTOMY ON INSULIN ACTION.

Houssay's experiments have shown clearly that hypophysectomized dogs develop a greatly increased sensitivity to the hypoglycæmic action of insulin. This statement we can fully confirm from our observations on rabbits and, further, in agreement with Houssay, we have found that hypophysectomized animals show a definite tendency to develop spontaneous hypoglycæmia. This complication has been the greatest danger in the present series of experiments. The rabbits quickly recovered from operative shock, but it was not an uncommon experience to find animals in profound hypoglycæmia despite the fact that food had not been withdrawn. In cases where liver glycogen studies were required we had to impose a preliminary period of fasting, but found that it was unsafe to extend this period beyond 12 hours. Even then, we lost some rabbits on which preliminary tests had been carried out. In animals developing spontaneous hypoglycæmia, from which we managed to obtain samples just at the pre-moribund stage, we invariably found a greatly depleted liver glycogen content.

As a starting point we decided to investigate the effect of hypophysectomy on the responses elicited by insulin, adrenaline and glucose administration. For this purpose we tabulated the responses to these substances in a series of twelve normal rabbits. At a later stage the animals were hypophysectomized and, at an interval varying from 10 to 14 days, the tests were repeated. In the case of normal animals two or three tests were carried out with insulin, adrenaline and glucose administration. The results were sufficiently uniform to justify a series of similar tests after operation. Although we could carry out repeated tests with glucose and adrenaline administration in hypophysectomized rabbits, we soon found that this was quite impossible with insulin. These operated animals soon developed an extraordinary sensitivity to insulin, and, when once hypoglycæmic symptoms had developed, it was practically impossible to avert a fatal issue.

The following experiments on Rabbit R 3 will illustrate the behaviour of most completely hypophysectomized rabbits:

*Rabbit R 3.* Wt. 3 kg. Injection in each case 0.5 unit insulin.

Before hypophysectomy (24 hr. fast): Initial blood sugar 98 mg. p.c. Blood sugar at hourly intervals after insulin—74, 86, 104, 114, 111 mg. p.c.

After hypophysectomy (12 hr. fast): Initial blood sugar 98 mg. p.c. 1 hr. after insulin—33 mg. p.c.; severe hypoglycæmic convulsion.

It will be observed that, before hypophysectomy, 0.5 unit of insulin produced a mild hypoglycæmic reaction from which the animal rapidly recovered, so that the effect had completely disappeared within 3 hours of the insulin injection. After hypophysectomy, on the other hand, a severe hypoglycæmia had developed within an hour of the insulin injection; the ensuing convulsions, although temporarily relieved by an intravenous injection of 1.25 g. glucose, returned within an hour and were again relieved by a second injection of glucose. An hour later the convulsions recurred with greater severity, and were no longer relieved by glucose injection. The animal was therefore killed and samples of liver taken for glycogen estimation. From our earlier experiences with animals developing spontaneous hypoglycæmia, we rather expected to find a greatly depleted liver glycogen in R 3. To our great surprise the value found was 1.6 p.c. It is important to note that a small, usually non-convulsant, dose of insulin produced a rapid and severe hypoglycæmia from which it was impossible to restore the animal by glucose administration. It is common experience that hypoglycæmia produced by much larger doses of insulin in normal rabbits can be readily relieved by glucose or adrenaline administration.

From the behaviour of R 3 and of other operated rabbits, which showed after death liver glycogen values of 2.8 and even 6 p.c., we can state definitely that the increased sensitivity to insulin is not due to depletion of liver glycogen. We have also observed a striking hypersensitivity to insulin in young hypophysectomized rabbits (see Table II). In this experiment a normal rabbit fasting 24 hours was given 1 unit of insulin and became convulsed  $2\frac{1}{2}$  hours later. A hypophysectomized litter mate received 0.2 unit and became convulsed  $1\frac{1}{2}$  hours later.

Owing to the normally wide variations found in adult rabbits, we cannot state that there is a clearly significant difference between glycogen values found in normal and in hypophysectomized animals, except in the case of the low glycogen content found in the livers of operated rabbits which develop spontaneous hypoglycæmia. Although, however, the fasting values for liver glycogen in hypophysectomized rabbits fall

within normal limits, the liver weight is greatly decreased [White, 1933], so that there is obviously less total liver glycogen present in the operated animals. Nevertheless, from actual calculations, we are certain that the total glycogen available, although on the average it is lower, is not reduced to anything like the extent found in those animals which developed spontaneous hypoglycæmia. When, in addition, glucose is given to the animal by mouth shortly before the insulin injection, we may be reasonably certain that the liver contains an adequate amount of glycogen. Even under these conditions, however, a small dose of insulin may produce a severe hypoglycæmia, as illustrated by the following experiment:

*Rabbit R 9.* Wt. about 2.5 kg. Injection in each case 0.5 unit insulin.

Before hypophysectomy (24 hr. fast): Initial blood sugar 120 mg. p.c. Blood sugar at hourly intervals after insulin—77, 62, 69, 65, 110 mg. p.c.

28 days after hypophysectomy (12 hr. fast): Initial blood sugar 80 mg. p.c., 5 g. glucose in 20 c.c. water was given by mouth. 1 hour later the blood sugar had risen to 143 mg. p.c. and in another hour and a half had fallen to 124 mg. p.c., at which point the insulin was injected. One hour later the blood sugar had fallen to 63 mg. p.c., and in another half hour to 32 mg. p.c., with incidence of severe convulsions. The animal was killed, and the liver glycogen content found to be 2 p.c. Autopsy showed a complete pituitary removal.

The animal deprived of its pituitary body, even when given glucose, so that the liver contained plenty of glycogen, was thus much more sensitive to insulin than when tested in the fasting condition before the operation. The fact that some animals tended to develop spontaneous hypoglycæmic symptoms, while others were apparently not so susceptible to this complication though still showing a greatly increased sensitivity to insulin, could not be attributed to variations in the completeness of pituitary removal, since animals with complete hypophysectomy, verified by serial sections of the contents of the sella, were found in both groups.

#### THE EFFECT OF VASO-PRESSIN AND OF ADRENALINE IN RELIEVING INSULIN HYPOGLYCÆMIA IN HYPOPHYSECTOMIZED RABBITS.

The failure of glucose to effect more than a temporary restoration of hypophysectomized animals from insulin hyperglycæmia led us to investigate the action of other substances, such as vaso-pressin and adrenaline. Burn [1928] considers that vaso-pressin and not oxytocin is effective in combating insulin hypoglycæmia. The results shown below do not indicate that vaso-pressin is at all an effective agent in the animals under consideration.

*Rabbit R 2.* Wt. about 3 kg. Completely hypophysectomized. Fasting 12 hours.

10.50: 0.5 unit insulin.

11.35: Severe convulsion, given 50 units vaso-pressin subcutaneously. The hypoglycæmic symptoms were slightly relieved.

12.25: Severe convulsions—with slight recovery.

12.30: 40 units vaso-pressin subcutaneously—no relief of symptoms.

12.50: No abatement in severity of convulsions. Animal killed. Liver glycogen 2.86 p.c.

Apart from the failure of vaso-pressin to relieve the convulsions, this experiment is remarkable for the high glycogen content of the liver, even after a protracted insulin hypoglycæmia.

Our results with adrenaline showed a certain inconclusiveness. Even in some normal animals, adrenaline proved definitely inferior to glucose as an agent for relieving hypoglycæmic symptoms. In some hypophysectomized animals it failed to relieve symptoms, while in others it produced a slow recovery. On the whole, however, it was less effective in relieving hypoglycæmia in the operated than in otherwise normal animals. It seemed further desirable to test its effectiveness in the hypophysectomized rabbit in which we had ensured an adequate supply of liver glycogen by previous glucose feeding. The results of such an experiment, with a control on a normal rabbit for comparison, are shown below:

*Rabbit 15 a.* Wt. about 2.5 kg. Normal animal, fasting 24 hours.

Initial blood sugar 117 mg. p.c. Given 5 g. glucose orally at 9.30.

11.0: Blood sugar was 152.

12.30: Blood sugar 118; 10 units insulin subcutaneously.

1.30: Blood sugar 54; 0.5 mg. adrenaline subcutaneously.

1.55: Blood sugar 70.

2.15: Blood sugar 70.

It will be noted in this glucose-fed, normal rabbit that 0.5 mg. adrenaline checked within 25 min. the effect produced by 10 units of insulin. These normal glucose-fed rabbits were found to require at least 6–10 units of insulin to produce a definite hypoglycæmia. This is in marked contrast to hypophysectomized ones, which, despite glucose feeding, still show a severe reaction to 0.5 unit insulin.

*Rabbit R 25.* Wt. 2.2 kg. Operated upon 25 days previously. Fasting 12 hours.

10.30: Blood sugar 88 mg. p.c.; given 5 g. glucose orally.

11.30: Blood sugar 153.

1.30: Blood sugar 104; 0.5 unit insulin subcutaneously.

3.00: Blood sugar 25; at this point severe convulsions, which continued unrelieved by an injection of 0.5 mg. adrenaline.

The animal was finally restored by an intravenous injection of glucose.

In several other similar experiments adrenaline produced a slow but definite relief, though, as previously stated, its action was never as potent as that observed in most normal animals.

Histological observations by one of us [White] have demonstrated that hypophysectomized rabbits develop a marked atrophy of the adrenal cortex. The medulla, however, appears normal in structure and exhibits the typical chromophile reaction. Some adrenaline-content determinations on normal and hypophysectomized rabbits were kindly carried out for us by Dr Schild. Colorimetric and physiological assays were made. These results are shown in Table I.

TABLE I.

	Body wt. in kg.	Wt. of both suprarenals in mg.	Total adrenaline content in $\gamma$	Wt. of liver in g.	Liver glycogen p.c.
Completely hypophy- sectomized rabbits	2.9	130	< 80	42	1.34
	3.0	206	122	62	12
	3.3	205	55	65	11
	2.2	200	< 40	—	0.04
	4.2	220	110	—	—
Partially hypophy- sectomized rabbits	2.4	440	240	—	—
	3.8	278	160	—	10
Normal rabbits	3.1	405	85	—	—
	2.0	162	50	—	—
	3.1	522	63	—	—

There seems to be a wide variation among normal and hypophysectomized animals, and no consistent distinction between them; the results do not give any evidence of adrenaline insufficiency following hypophysectomy.

#### GLUCOSE TOLERANCE AND ADRENALINE HYPERGLYCÆMIA IN HYPOPHYSECTOMIZED RABBITS.

In a few cases no marked differences were observed before and after operation. Most animals, however, showed a definitely increased glucose tolerance. From experiments carried out in metabolism cages this was shown not to be due to a lowered renal threshold for glucose with attendant glycosuria. The oral administration of 5 g. glucose in 20 c.c. water is usually followed by a moderate hyperglycæmia, and at the end of  $2\frac{1}{2}$ –3 hours the blood sugar is still slightly above the fasting level. In most hypophysectomized animals the hyperglycæmia was distinctly decreased, and in some instances the blood sugar at the end of  $2\frac{1}{2}$ –3 hours was definitely below the fasting level. Practically the same remarks apply to the hyperglycæmic response produced by 0.2 mg. adrenaline.

Thus after a preliminary small rise the blood sugar tended to fall below the fasting value. The following results, all obtained on the same rabbit R 3, illustrate these statements:

*Rabbit R 3.* Given 5 g. glucose by mouth.

Before hypophysectomy (24 hr. fasting): Initial blood sugar 93 mg. p.c. Blood sugar at half-hourly intervals after glucose—180, 220, 230, 220, 190 mg. p.c.

After hypophysectomy (12 hr. fasting): Initial blood sugar 97. After glucose—92, 108, 104, 111, 104 mg. p.c.

*Rabbit R 3.* 0.2 mg. adrenaline subcutaneously.

Before hypophysectomy (24 hr. fasting): Initial blood sugar 115 mg. p.c. Blood sugar at half-hourly intervals after adrenaline—130, 155, 130, 115, 105 mg. p.c.

After hypophysectomy (12 hr. fasting): Initial blood sugar 96. After adrenaline—113, 108, 99, 90, 72 mg. p.c.

The response of this rabbit to insulin has already been discussed.

The absence of a normal adrenaline response cannot be attributed to a depleted store of liver glycogen, since it is also observed after a preliminary glucose feeding, just as is the abnormal insulin response.

#### THE EFFECT OF INSULIN ON LIVER GLYCOGEN STORAGE IN YOUNG HYPOPHYSECTOMIZED RABBITS.

The young rabbit is unique in that it is the only animal which shows a regular and definite deposition of liver glycogen after small doses of insulin. Investigations of this phenomenon by one of us [Corkill] led to the suggestion that it was not due to an uncomplicated effect of insulin, but in all probability involved the action of adrenaline, and possibly other hormones, secreted in response to the insulin hypoglycæmia. More recent investigations, shortly to be published, in which the effect of ergotoxine has been tested, have raised a doubt as to the significance attributed to adrenaline. See also Goldblatt [1933].

In view of the possible participation of hormones from the pituitary body, it seemed of interest to study this action of insulin in young hypophysectomized rabbits. As already mentioned, operation in young rabbits is liable to be attended by a high operative mortality, so that it was impossible to obtain a large series of animals. Apart from operative trauma, our greatest loss was due to severe spontaneous hypoglycæmic symptoms, which were apt to appear within 48 hours of the operation. We managed, however, to obtain several animals which, 5 days after the operation, appeared to be fully recovered, and showed no obvious abnormality.

In Table II the values obtained on two separate litters are combined. The general plan of the experiment and chemical estimations followed that already described in a previous paper [Corkill, 1930].

TABLE II.

	Glycogen p.c.	
	Liver	Muscle
1. Normal control	0.30	0.34
2. Normal control	0.48	0.26
3. Normal, injected with 1 unit insulin, convulsed 3½ hr. later	2.55	0.24
4. Hypophysectomized control	0.16	0.15
5. Hypophysectomized, injected with 0.2 unit insulin, convulsed 1½ hr. later	0.45	0.13

In this single experiment the hypophysectomized animal showed a greatly increased sensitivity to insulin. Even though the dose was greatly reduced, so that hypoglycæmic symptoms did not occur until 90 min. later, this animal did not show any definite deposition of liver glycogen, the values obtained being clearly within normal limits.

#### DISCUSSION.

If we accept Houssay's main findings, and these appear to be based on careful experimental work, we must assume that the anterior lobe of the pituitary body influences carbohydrate metabolism in a sense directly opposed to that of the internal pancreatic secretion; for hyperfunction of the anterior lobe, or repeated injection of its extract, is followed by symptoms characteristic of deficient pancreatic function, namely diabetes; while, on the other hand, ablation of the anterior lobe produces a condition in many ways suggestive of excessive pancreatic function, and we might well expect that, under such conditions, the additional effect of quite a small injection of insulin would lead to the development of a severe hypoglycæmia. But the characteristic hypersensitiveness to insulin of hypophysectomized animals, with which we are here particularly concerned, cannot be due merely to an excessive secretion of insulin by the pancreas, since this would not explain why, in the absence of the pancreas, removal of the pituitary body abolishes the specific symptoms of insulin deficiency.

For a clear appreciation of the problem, it is important to remember that the course of the blood sugar after administration of insulin is determined jointly by the effect of the insulin, and by the compensatory response of the organism, which tends to restore the lowered blood sugar to the initial level. Thus there is good reason to believe [Cannon, *et al.* 1924] that when the blood sugar has fallen to a certain critical level, usually about 75 mg. p.c., a compensatory secretion of adrenaline by the suprarenals takes place, which in turn causes a discharge of glycogen from the liver and a consequent replenishment of the lowered blood



sugar. One of the commonest causes of hypersensitivity to insulin is a derangement of this compensatory mechanism, and this would seem to be effective in the present case, in view of the observed difficulty with which the animals recover from the hypoglycæmic effects of insulin.

Obviously if the glycogen reserves in the liver are depleted, the blood sugar will fail to rise. In the present case, however, such an explanation is inadmissible, for the hypersensitive rabbits with which we worked did not show evidence of glycogen depletion until the final stages of spontaneous hypoglycæmia. Even when we had ensured a plentiful supply of liver glycogen by previous administration of glucose, the hypersensitivity to insulin was still in evidence.

We must therefore look elsewhere for the breakdown in the compensatory mechanism. It is well known that removal of the pituitary body leads to a marked decrease in size of the suprarenal glands, due mainly to a degenerative shrinkage of the cortical tissue. It seemed possible, however, that the medulla might also be affected, and that the cause of the breakdown might lie in the presence in the medulla of insufficient adrenaline for the purposes of glycogen mobilization. Our comparisons of the adrenaline content of the suprarenals of normal and hypophysectomized rabbits do not, however, support this view; nor have we any reason to suspect a defective liberation of adrenaline from the glands, in response to the hypoglycæmic stimulus.

Further, whatever its cause, if lack of "available" adrenaline were responsible for the impaired recovery of hypophysectomized animals, we should expect a rapid recovery to occur when the requisite adrenaline was supplied from without. This, however, was not the case. It appears, then, that the failure of the hypophysectomized rabbit to recover from insulin hypoglycæmia is not due to the lack either of liver glycogen or of adrenaline to mobilize it, for it is still observed even when both glycogen and adrenaline are available in adequate amount. We must therefore suppose that the liver glycogen has become abnormally resistant to the mobilizing action of adrenaline, and this supposition is borne out by the impaired ability, and sometimes complete failure, of adrenaline to produce hyperglycæmia in hypophysectomized animals.

This picture of increased response to insulin and diminished response to adrenaline somewhat resembles that observed by Burn and Marks [1925] in rabbits deprived of the thyroid gland, and it may well be due in part to the degeneration of the thyroid gland, known to take place after removal of the pituitary body. The loss of resistance to insulin intoxication in the absence of the pituitary gland, however, is far more

severe than anything observed in thyroidectomized animals, and there is probably some further endocrine deficiency which aggravates the condition of the hypophysectomized animal. In view of the extreme atrophic changes in the adrenal cortex, we are tempted to suspect that cortical deficiency may play an important part. Although Britton and Silvette [1932] have published some suggestive observations, the rôle of the adrenal cortex in carbohydrate metabolism is still obscure. We are carrying on concurrently an investigation on adrenalectomized animals, which we hope to link up with the work reported here.

#### SUMMARY AND CONCLUSIONS.

1. Rabbits from which the pituitary gland has been removed became abnormally sensitive to the hypoglycæmic action of insulin, and may even develop a spontaneous hypoglycæmia, especially when deprived of food for several hours.

2. In animals which exhibit spontaneous hypoglycæmia the glycogen reserves are found to be depleted, and this lack of available carbohydrate may be a contributory cause to the fall in blood sugar.

3. Lack of carbohydrate is not responsible for the increased response to insulin, for the latter is observed in animals which have ample reserves of liver glycogen.

4. The increased insulin response is characterized by delay in the return of the blood sugar to the normal level. The hypoglycæmic symptoms are usually severe and are relieved only with great difficulty. Injections of adrenaline or of vaso-pressin, which will usually relieve insulin hypoglycæmia in the normal animal, have little or no effect in the animal deprived of its pituitary body.

5. Animals which are abnormally sensitive to insulin usually exhibit also a diminished response to adrenaline, and an increased sugar tolerance. It is suggested that an abnormal resistance of the glycogen reserves to the mobilizing action of adrenaline is a factor in the increased sensitiveness to insulin.

6. The possibility that this stabilization of liver glycogen is consequent upon the thyroid degeneration observed after removal of the pituitary body is being investigated.

7. Young rabbits, which normally deposit liver glycogen as a result of insulin injection, fail to do so when deprived of the pituitary gland.

We wish to thank Sir Henry Dale for his stimulating interest in this investigation.

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## A COMPARISON OF FŒTAL AND MATERNAL HÆMOGLOBINS IN THE GOAT.

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(*Received September 19, 1933.*)

RECENT investigations have shown that the oxygen dissociation curves of foetal and maternal bloods are not identical. Comparisons on the blood of goats have been published by Huggett [1927] and on human blood by Haselhorst and Stromberger [1931]. The affinity of hæmoglobin for oxygen depends upon the concentrations of hydrogen ions and other electrolytes, and in order to determine whether the differences in the dissociation curves are due to differences in the hæmoglobins, experiments have been made in this work on purified solutions of maternal and foetal hæmoglobins, in which the concentrations of hydrogen ions and salts have been defined by the dialysis of the protein solutions against relatively large volumes of a standard buffer solution. In addition, measurements of osmotic pressure and of the membrane potential have been made in order to determine whether there is any difference between the molecular weights or the ionization of maternal and foetal hæmoglobins<sup>1</sup>.

*Preparation of material.* Samples of the blood of maternal and foetal goats were used for the preparation of hæmoglobin. The corpuscles were washed three times with salt solution. The stromata were removed by the use of purified ether and sodium chloride, as described by Adair [1925]. The preparations were placed in collodion membranes and dialysed for 1 day with distilled water and then for periods from 4 to 10 days with the Sørensen phosphate buffer mixture containing 1/30 mol. of potassium dihydrogen phosphate and 1/30 mol. of disodium hydrogen phosphate per litre of solution. The pH value of this mixture is 6.81 at 20° and about 6.78 at 38° C. For the purposes of this work it seemed desirable to use the solution at pH 6.8 rather than at the physiological 7.4, or more alkaline reactions. In the first place, higher oxygen tensions are used for the

<sup>1</sup> Dilute solutions of hæmoglobin from the same series of goats were studied simultaneously by Prof. F. G. Hall using spectroscopic methods. The results were similar to those found in this paper and will be published shortly.

dissociation curve at 6.8, and the oxygen pressure at half saturation can be determined with greater accuracy from the gas analyses. A second and more important advantage is that the *pH* values of different hæmoglobin preparations are less variable after dialysis with solutions at *pH* 6.8 than at 7.4, because the phosphates have a higher buffer value at 6.8 and are less affected by traces of carbon dioxide. The effects of variations in the *pH* on the oxygen dissociation curve are smaller at *pH* 6.8 than at 7.4. Even if the *pH* values are identical at 0°, there may be variations at 38°, because the effect of temperature on the *pH* may be greater in the solutions with higher concentrations of hæmoglobin. The temperature correction is less important at *pH* 6.8 than at 7.4.

There is one disadvantage in working at *pH* 6.8; oxyhæmoglobin changes into methæmoglobin more rapidly at *pH* 6.8 than in more alkaline solutions. Estimates of the methæmoglobin formation were made by the following procedure. The refractive indices of the protein solution *R'* and of the dialysate *R''* were measured and the protein concentration *C* was calculated by the formula  $R' - R'' = 0.001945 C$  as described by Stoddard and Adair [1923]. The theoretical oxygen capacity was calculated on the provisional assumption that 1 g. of the goat's hæmoglobin is equivalent to 1.34 c.c. of oxygen, the value accepted for the hæmoglobin of the horse. The oxygen capacities were then determined and the results are recorded in Table I. Provisional estimates of

TABLE I.

	Date of pregnancy, weeks and days	Date dialysis started	Date dialysis ended	Date dissociation curve measured	Oxygen pressure in mm. at half saturation	<i>n</i>	Oxygen p.c. of theoretical capacity	Oxygen retical
Normal	—	March 25	March 31	April 7	47	2.12	14.01	92.3
Maternal	12	April 19	April 26	April 26	43	$2.04 \pm 0.2^*$	12.7	85.3
Maternal	13, 2	April 25	May 3	May 27	36	$2.2 \pm 0.2^*$	8.59	90.5
Fœtal	13, 2	April 24	May 2	May 2	31	1.7	6.76	82.8
Maternal	15, 1	May 5	May 10	May 19	39	$2.25 \pm 0.15^*$	13.36	84.7
Fœtal	15, 1	May 5	May 10	May 15	31.5	2.17	8.98	85.2
Maternal	16	June 1	June 8	July 22	42	2.35	6.87	74.7
Fœtal	16	June 1	June 8	July 13	24.5	2.17	7.18	—
Maternal	18	May 23	June 2	June 13	38	2.0	8.50	86.6
Fœtal	18	May 22	June 2	June 7	22.5	—	4.60	72.8
Maternal	19, 6	June 1	June 8	July 18	34	2.27	14.1	71.5
Fœtal	19, 6	June 1	June 8	July 17	26	$2.5 \pm 0.3^*$	8.15	73.6

\* Probable error in the determination of *n*.

the percentage of the total hæmoglobin in the active form are recorded in Table I, column 9. These figures may require multiplication by a constant factor when goats' hæmoglobin has been subjected to further

analysis, but from the results of colorimetric determinations of total hæmoglobin it appears unlikely that the factor will exceed 1.05. It appears therefore that some of the preparations contain over 20 p.c. of methæmoglobin.

It is by no means certain that the methæmoglobin formation is wholly due to the  $pH$  of the buffer mixture. In a programme of work on blood as well as on hæmoglobin, the blood must be dealt with first in order to minimize the risk of changes in  $pH$  and it may be necessary to defer work on the hæmoglobin. There is little doubt that higher percentages of oxyhæmoglobin could be obtained if the work could be finished in a shorter time.

Methæmoglobin interferes with certain methods for the study of the dissociation curve. The experimental results described below indicate that there is little difference between the dissociation curves of solutions containing varying amounts of methæmoglobin. Since the amounts of methæmoglobin formed in maternal and foetal hæmoglobins, dialysed at the same time are the same, there is no reason to suppose that methæmoglobin formation vitiates the comparison of maternal and foetal hæmoglobins.

#### *Oxygen dissociation curves.*

For the measurement of the oxygen dissociation curves, the hæmoglobin solutions were equilibrated at  $37^{\circ}C$ . in the new Barcroft saturator<sup>1</sup> with an enclosed double bulb pipette. Duplicate Haldane analyses were made on the gas phase, and the percentage oxygenation of the solutions was determined by the van Slyke manometric method.

The foetal and maternal hæmoglobin solutions were obtained from goats in the 12th–20th week of gestation. The total period of pregnancy in the goat is 21 weeks. A non-pregnant goat was used as a control. The characteristics of the hæmoglobin solutions used are shown in Table I.

The oxygen dissociation curves of a number of preparations are shown in Fig. 1, and experimental data for the oxygen dissociation curves of additional preparations are recorded in Table II. The curve numbered 1 is a composite curve of all the maternal hæmoglobins. The range of variation covered by the points is comparatively small, and it appears that the oxygen dissociation curves of all the maternal hæmoglobins are nearly identical. The pair of curves, numbered 2 and 3, represent the foetal and maternal hæmoglobin obtained at the 16th week of pregnancy. The foetal and maternal hæmoglobins obtained at the 18th week of pregnancy are represented by curves 4 and 5, and those obtained at the 20th

<sup>1</sup> A description of this apparatus will be published later.

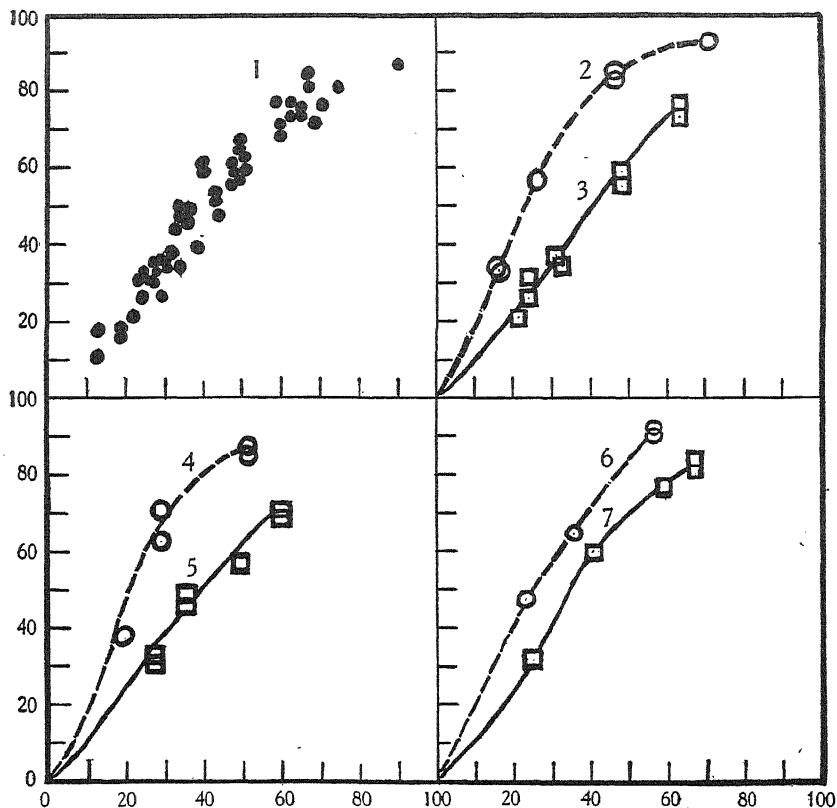


Fig. 1. Oxygen dissociation curves of hæmoglobin of goats. Ordinates: percentage oxygenation. Abscissæ: oxygen pressures, mm. of Hg. Circles: foetal. Squares: maternal. Interpretation in text.

TABLE II. Percentage oxygenation and oxygen pressures in mm. of Hg.

Normal	Pressure in mm.	33.6	38.7	44.2	69.0	—	—
	Saturation p.c.	34.0	39.0	47.0	70.8	—	—
Maternal, 12th week	Pressure in mm.	29.0	43.5	51.0	90.5	—	—
	Saturation p.c.	26.4	51.3	59.0	87.0	—	—
		35.6	53.0	62.0	—	—	—
Foetal, 13th week	Pressure in mm.	26.4	13.2	50.0	80.7	—	—
	Saturation p.c.	36.6	19.6	79.1	74.4	—	—
		45.8	21.4	66.0	85.9	—	—
Maternal, 13th week	Pressure in mm.	12.8	27.9	33.7	49.5	71.0	—
	Saturation p.c.	10.10	32.9	47.2	64.5	75.5	—
		17.85	34.5	49.5	67.0	76.5	—
Foetal, 15th week	Pressure in mm.	14.3	26.3	29.0	55.5	—	—
	Saturation p.c.	13.0	37.4	42.2	78.0	—	—
		16.3	38.0	44.5	73.0	—	—
Maternal, 15th week	Pressure in mm.	19.3	30.3	32.8	65.4	48.0	75.2
	Saturation p.c.	15.9	34.3	44.3	73.5	60.7	80.5
		17.5	35.4	44.6	75.0	—	—

week by curves 6 and 7. The goat from which these curves (6 and 7) were obtained died of asphyxia during operation. Subsequent investigation revealed a condition of pulmonary congestion.

It is evident that in all cases the curve for foetal hæmoglobin is on the left of the maternal, so that foetal hæmoglobin has a higher affinity for oxygen. In the second place there seems to be a slight difference in the shape of the curves, which can be represented by the calculation of  $n$  in Hill's equation,  $y/100 = kx^n/(1 + kx^n)$ , in which  $y$  represents percentage saturation,  $x$  oxygen tension and  $k$  is a constant. The mean value of  $n$  for the maternal hæmoglobins is 2.2 with a probable error of  $\pm 0.3$ , and for foetal hæmoglobins 2.0 with an error of  $\pm 0.4$ . It is open to question whether the difference is significant, in view of the wide range of error in the determination of  $n$ , but it may be of interest, because a similar difference has been obtained in the bloods<sup>1</sup>.

Human foetal and maternal bloods have been studied by Haselhorst and Stromberger [1931]. They determined the relationship between the  $k$  of Hill's formula and the hydrogen-ion concentration, assuming that  $n$  is a constant. Although it is not possible to compare the values of  $n$  for the human hæmoglobins, the values of  $k$  for the human foetal hæmoglobin are higher, in accordance with observations on goat's blood.

The observations recorded in this paper indicate that the differences in the bloods are due to differences in maternal and foetal hæmoglobin rather than to any possible differences between the electrolytes and other substances present in maternal and foetal corpuscles.

#### *The osmotic pressures of maternal and foetal hæmoglobins.*

In view of the difference between the dissociation curves of maternal and foetal hæmoglobins, a number of measurements of the osmotic pressure of the proteins were made, using the same phosphate buffer mixture. Osmometers with collodion membranes were used as described by Adair [1925]. The protein concentrations were determined by the refractometric method. The observations are recorded in Fig. 2. The continuous curve in Fig. 2 represents the osmotic pressure of the hæmoglobin of the sheep [Adair, 1928]. The circles represent observations on the foetal hæmoglobin of the goat, and the squares on the maternal hæmoglobin. It appears that both of these forms have osmotic pressures in fairly close agreement with the data for sheep's hæmoglobin, which has a molecular weight of 68,000. Most of the points for foetal hæmoglobin lie above the curve, but it is not unlikely that the relatively small

<sup>1</sup> Observations on blood made in this laboratory.



differences observed are due to experimental error. The results in Fig. 2 may be subject to a slight error, because the calculations of the protein concentration depend upon the assumption that the refraction value of a 1 p.c. solution is 0.001945, as in the case of the hæmoglobin of the sheep.

In addition to these measurements of osmotic pressure, a number of measurements of the membrane potential have been made, and from these the ratio  $E/C_v$  has been calculated as described by Adair and

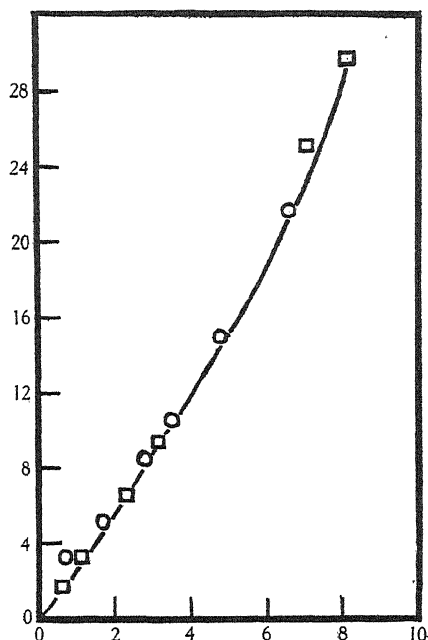


Fig. 2. Ordinates: osmotic pressure in mm. of mercury at 0° C. Abscissæ: concentrations in grams of protein per 100 c.c. of solution. The smooth curve is copied from the curve for sheep's hæmoglobin given by Adair [1928]. The circles are fœtal hæmoglobin, squares maternal hæmoglobin.

Robinson [1930].  $E$  represents the membrane potential in millivolts and  $C_v$  the corrected concentration of the protein in grams per 100 c.c. of solvent. In the case of the maternal hæmoglobin the following values of  $E/C_v$  were obtained:

0.057, 0.055, 0.058, 0.075, 0.057.

In the case of fœtal hæmoglobin the ratios obtained were:

0.099, 0.077, 0.105, 0.091.

In all cases the protein solutions were negative, the protein acting as an anion in the buffer mixture at  $pH$  6.9 at  $0^{\circ} C$ . It appears that the foetal hæmoglobin has more negative charge than the maternal hæmoglobin at the same  $pH$ , but the potentials observed were so small that the difference might possibly be due to experimental error.

#### SUMMARY.

1. Comparative investigations have been carried out on purified solutions of foetal and maternal hæmoglobin of goats. Oxygen dissociation curves obtained from these solutions have shown that foetal hæmoglobin has a greater affinity for oxygen than maternal hæmoglobin.

2. Determinations of osmotic pressure revealed no appreciable difference between the molecular weights of foetal and maternal hæmoglobins.

This work forms a part of a study of the conditions of foetal respiration planned by Prof. Barcroft, and I wish to thank him for his kind advice and help. I also wish to express my gratitude to Mr G. S. Adair for his generous and invaluable assistance in supervising the dialysis of solutions and the osmotic pressure determinations.

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## THE EFFECT OF MUSCLE LENGTH ON THE ENERGY FOR MAINTENANCE OF TENSION.

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It has been shown by Hill [1925] that the energy liberated by a muscle during a short tetanus can be analysed into heat for the development of tension and heat for the maintenance of tension. Thus the initial heat produced in a 0.3 sec. tetanus is greater than that produced in a 0.1 sec. tetanus by an amount which represents the energy needed to maintain tension for the last 0.2 sec. Hill [1925] has also shown that the initial heat in a twitch, or the energy needed to develop tension, is at a maximum at a muscle length corresponding roughly to 90 p.c. of the resting length of the muscle, while the tension developed by the muscle is at a maximum at the resting length. It is the purpose of this paper to show that the energy to maintain tension is affected by changes in the length of the muscle in the same manner as the energy to develop tension.

The behaviour of this "maintenance energy" at different muscle lengths has a certain theoretical importance which prompted the present investigation. It was shown by Fenn [1923] that an extremely small shortening (3 p.c.) of a muscle in an otherwise isometric contraction may lead to a considerable (18 p.c.) increase in heat production, and, further, that the internal work in a gastrocnemius muscle due to the inevitable shortening of the fibres, even when the muscle as a whole is rigidly isometric, causes enough extra heat to account for the dissimilarity in the heat production of the sartorius and gastrocnemius muscles in isotonic contractions. Even in a sartorius muscle, however, the "isometric" contractions involve some internal work which no one has yet even attempted to evaluate and which may be of great importance, particularly when isometric heats at different lengths are compared. The internal work certainly would vary with the length of the muscle and

might account in part, or even *in toto*, for the variations of heat with length of muscle. If this were the case, then presumably the energy for maintenance of tension would not vary with the length of the muscle, as does the energy for the development of tension, for it is liberated after the internal work is over.

#### METHOD.

Single sartorius muscles from *Rana pipiens* were used as material. The thermopile was one designed by Bozler [1930] for use with smooth muscle and had a single row of hot junctions. The upper, tibial end of the muscle was fastened to the isometric lever by a fine drill rod to avoid stretching. The isometric lever was of the torsion wire type and was equipped with an extra writing point fixed to the frame to record zero tension. The increase in length of the muscle was measured from the amount by which the lever was raised. The resting extended length of the muscle in the frog was measured in most experiments and found to correspond fairly well with the length for maximum development of tension. The thermopile chamber was kept immersed in a "thermos" jug at room temperature. The thermopile was equipped with three electrodes, one near each edge of the thermopile and one near the upper end of the muscle. The middle and bottom electrodes were used for calibration and the top and bottom for stimulation. A.C. house current (60 cycles per sec.) cut down by a transformer and potentiometer was used for stimulation, duration being measured by a rotary contact breaker. Calibration was carried out mostly by Bozler's [1931] condenser method as applied by A. V. Hill [1931]. Heat was calculated for that part of the muscle lying between the middle and bottom electrodes, no correction being made for the muscle beyond the electrodes. The length of muscle thus considered was 2.5 cm. long.

The stimulus was adjusted to be just maximal as judged by the tension developed. Observations began at the shortest length, the heat for 0.1 and 0.3 sec. tetanus (or 0.2 and 0.4 sec.) being measured. Then the muscle was stretched in successive steps of 3 mm. each and stimulated for 0.1 and 0.3 sec. at each step until the resting tension began to increase considerably and the total tension development began to decrease. Finally the same series was repeated in the reverse order. If a good return series was not obtained the experiment was discarded. The "up" and "back" series were averaged together. Nine successful experiments were performed giving twenty series.

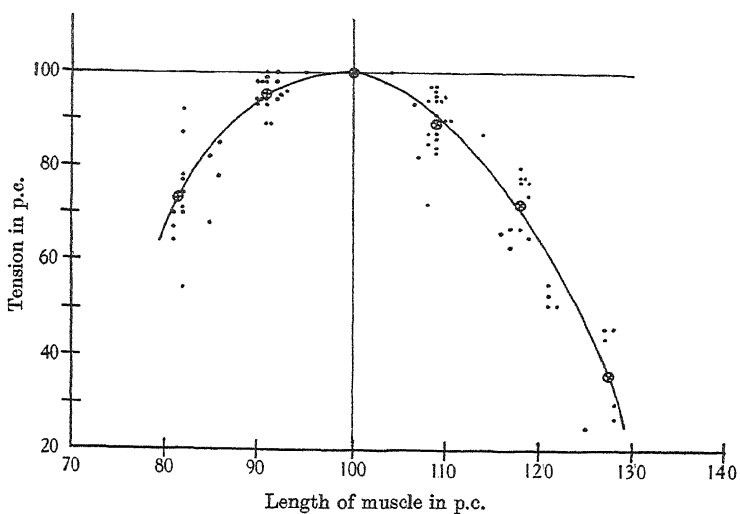


Fig. 1. Tension in p.c. of the maximum at different muscle lengths.

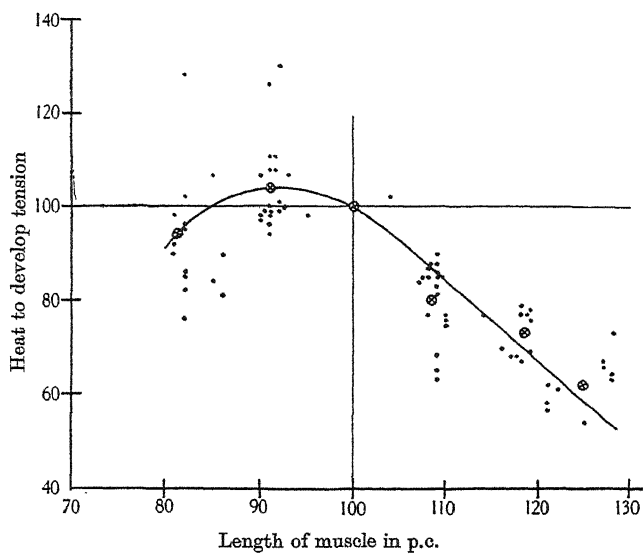


Fig. 2. Heat for the development of tension at different muscle lengths.

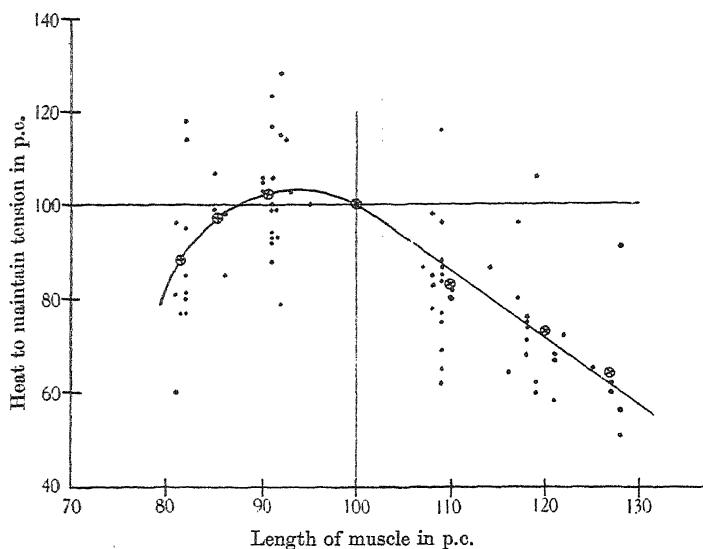


Fig. 3. Heat for the maintenance of tension at different muscle lengths.

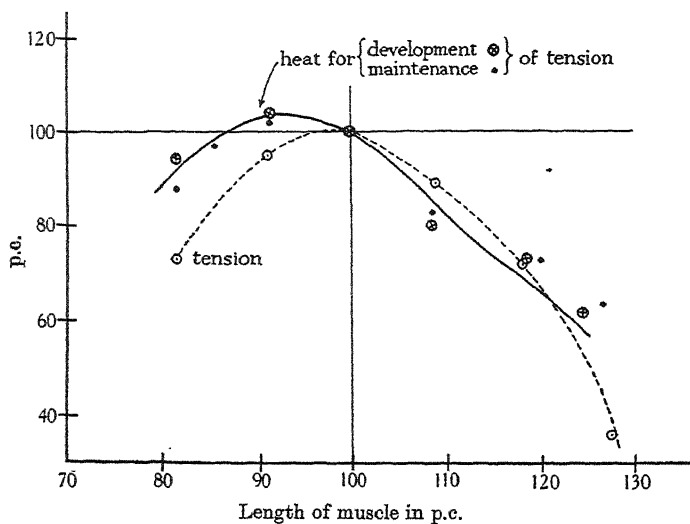


Fig. 4. Average points from Fig. 1-3 plotted together for comparison.

## RESULTS.

For purposes of presentation all the muscle lengths have been expressed in percentage of the resting length, the resting length being taken as the length at which the tension development is a maximum. Likewise the tension and heat developed are expressed in percentage of the tension and heat developed at this resting length.

The results are plotted in Figs. 1-4. Fig. 1 shows the tension developed plotted against the length of the muscle. All the points of all twenty series are plotted. The curve is drawn through average points marked by

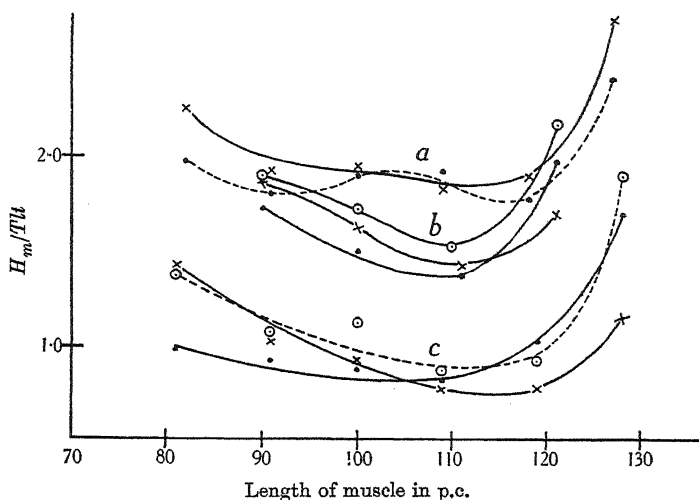


Fig. 5. Ratio of heat for the maintenance of tension (per cm. per sec.) to the tension maintained (or  $H_m/Tlt$ ) at different muscle lengths.

circles. The curve is similar to one obtained by Hill [1925]. Figs. 2 and 3 show curves similarly plotted for the heat to develop tension,  $H_d$  (0.1 or 0.2 sec. tetanus), and for the heat to maintain tension,  $H_m$  (heat for 0.4 sec. minus heat for 0.2 sec.). Both show a maximum at about 90 p.c. of the resting length (in exact agreement with Hill's heat curves), the scatter of the points being somewhat greater than for the tension. For purposes of comparison all three curves are plotted together in Fig. 4. The heat curves for maintenance and for development of tension are identical within limits of error of the method. This shows that the conditions, whatever they may be, which cause the heat of a twitch (or the

heat to develop tension) to vary with muscle length must obtain equally during the maintenance of tension. The heat associated with the varying internal work at different lengths is therefore presumably not involved unless there is some internal work due to alternate contraction and relaxation of the ultimate contractile units within the individual muscle fibrils during the maintenance of tension in a tetanus<sup>1</sup>.

The heat necessary to maintain 1 g. of tension in 1 cm. of muscle for 1 sec.,  $H_m/Tlt$ , has been calculated for all these experiments. Inspection of Fig. 4 shows that  $H_m$  exceeds  $T$  (both expressed in percentage) at very short and very long muscle lengths indicating that more heat is needed at these points for the maintenance of the same tension. Least heat is needed or greatest "economy" of tension maintenance is attained at lengths between 100 and 120 p.c. where tension exceeds heat. Thus the ratio  $H_m/Tlt$  for "maintenance heat" varies to some extent with the length of the muscle just as Hill found for the ratio  $H_a/Tl$  for the twitch.  $H_m/Tlt$  has a minimum value at a relative length of 110 p.c., which varies in different experiments from 0.75 to 1.9 g. cm. per g. tension per cm. length of muscle per second. Hartree and Hill [1921] give figures of 0.92 at 20° C. and 1.4 at 25° C. for spring English frogs (Table III of their paper). Hill found also later [1925], in general agreement with our results, that the ratio  $H/T$  passes through a minimum at the resting length of the muscle. The values of  $H_m/Tlt$  for three different muscles, *a*, *b*, and *c*, including eight series of increasing and decreasing lengths, are plotted in Fig. 5 to show the minimum mentioned, the slopes of the curves and the variations met with.

#### SUMMARY.

The energy necessary to maintain 1 g. of tension in 1 cm. length of frog's sartorius muscle for 1 sec. has been measured at different muscle lengths. The energy for the maintenance of tension is found to vary with changes in muscle length in exactly the same way as the energy for the development of tension. This would indicate that variations in internal shortening of the fibres at different lengths do not account for the variations in heat observed, since such shortenings are presumably all complete when the maintenance heat is being measured.

<sup>1</sup> It may be necessary to consider this apparently remote possibility seriously, for an estimate of the amount of internal work at different muscle lengths suggests that it is large enough to explain the variation of heat with length. This work, however, is not yet complete.



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THE PHOSPHORUS DISTRIBUTION IN  
RESTING FLY MUSCLE.BY ERNEST BALDWIN AND  
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## INTRODUCTION.

REPRESENTATIVE members of nearly all the invertebrate phyla have now been examined for their phosphagen content [Eggletton and Eggletton, 1928; Meyerhof, 1928; Needham, Needham, Baldwin and Yudkin, 1932]. It has been established that creatinephosphoric acid is characteristic of the Vertebrata, while argininephosphoric acid is restricted to the Invertebrata. The two compounds have only been found to co-exist in the Echinoids and in the Enteropneusts. This is of especial interest, since the latter (Hemichordates) form a morphological link between the vertebrate and the echinoderm phyla. Another protochordate group, the Ascidians (Urochordates), have recently been examined by Kreps [1933], who finds creatinephosphoric acid and no argininephosphoric acid; they thus resemble *Amphioxus* (Cephalochordates).

It must be remembered that only in the case of one species (the crayfish, *Astacus fluviatilis*) has arginine phosphoric acid actually been prepared from invertebrate muscle and analysed [Meyerhof and Lohmann, 1928]. In most cases the evidence for its presence consists in the demonstration of a phosphorus compound, stable in strong acid in presence of molybdate, but hydrolysing in 18 hours at 28° C. in *N*/20 acid in absence of molybdate. In the cases of *Pecten*, *Holothuria* and *Sipunculus*, Meyerhof has supported this evidence by showing that the base of the phosphagen (after hydrolysis) is attacked by arginase with production of urea. It is thus evident that much more work is needed on invertebrate phosphagen. Baldwin [1933] has brought forward evidence suggesting that the cephalopod phosphagen has different properties from argininephosphoric acid, and Arnold and Luck [1933]

have pointed out the possibility that a hitherto unknown phosphagen is contained in the polychaete worms.

Curiously enough, one large and important class of Invertebrata had, until very recent times, remained untouched—namely, the Insecta. The wing muscles of the flying insects are of special interest, as their rate of contraction and relaxation is said to be quite unparalleled in the animal kingdom. According to Marey [1873] in the house-fly the wings make 330 complete vibrations per second. We therefore took up the study of insect phosphagen, choosing *Calliphora* and *Lucilia* as good representatives of the flying insects in which rapidly acting musculature has reached its highest development. Besides this object, we also had the purpose of investigating the distribution of the whole acid soluble phosphorus and of following the changes in the fractions during fatigue and rigor. This had been done in a systematic manner only for the frog and the rat. The present paper describes the results on resting muscle; the work on stimulated muscle and on muscle in rigor is in progress. When this work was nearly finished, a paper by Schütze [1932] appeared; he had examined various insect muscles (*Locusta*, *Dytiscus*, *Hydrophilus*, *Lucanus*, *Apis*, *Aeschna*), and reported the presence of argininephosphoric acid, relying, however, only on the evidence from rate of hydrolysis.

The flies used in our work were raised from larvæ which were placed in suitable cages in a warm, well-lighted room. Pupation, hatching and breeding took place in due course; the adults were fed on sugar, water and liver. The insects were collected for the experiments in tall narrow beakers; this was easily accomplished by darkening the whole cage, applying the beaker to an opening, and illuminating the end of the beaker with a bright lamp. When enough insects had entered the beaker, the latter was covered by means of a Petri dish and entirely surrounded for some minutes in a freezing mixture. This treatment rapidly immobilized the insects, which were then tipped out into a small, cooled beaker to await dissection. We have assured ourselves that this treatment need not injure the flies; for if a few of the cooled insects were allowed to warm up, after not too long a time at the low temperature, rapid and apparently complete recovery followed. It was not feasible to dissect out the thoracic muscles individually, but as the thorax consists almost entirely of muscle, surrounded by a thin chitinous covering, this whole region was taken for analysis.

The amount of skeletal tissue in the thoraces was estimated by Buxton's method [1932]. The thoraces were dried at 100° C., finely powdered, then heated in a boiling water bath, under a reflux, for 5 hours

with 1 p.c. KOH. The contents of the flask were filtered through a weighed Gooch crucible, which, after washing, was dried and reweighed. From 3.3 g. of fresh tissue (150 flies) 0.119 g. of dry residue was obtained; so that 3.6 p.c. of the weight of fresh thorax is to be regarded as consisting of skeletal tissue. It was not possible to estimate the weight of the thoracic viscera, but it is quite clear from the data given by Lowne [1890-2] (see especially Plate XI) that it is very small in comparison with the muscle weight, certainly not more than about 5 p.c. of the fresh thorax weight. All the results are expressed in mg. per 100 g. of fresh thorax.

When the insects were completely motionless, they were placed, a few at a time, on a cooled glass plate, and the head, abdomen, wings and legs were dissected off from each. The thoraces were extracted at 0° C. by grinding thoroughly with ice-cold 5 p.c. trichloroacetic acid (usually about 5 c.c. per g. of tissue) and quartz sand. The extract was then filtered under pressure into a cold centrifuge tube and the clear, sparkling, slightly yellow filtrate was used for the experiments described below.

All the phosphate estimations were done by Fiske and Subbarow's method [1929], using a Klett colorimeter. The separation of the phosphate fractions into those with insoluble calcium and barium salts (free phosphate and adenylypyrophosphate) and those with soluble calcium and barium salts (phosphagen and hexosemonophosphate) was performed sometimes by means of Fiske and Subbarow's  $\text{CaCl}_2$ — $\text{Ca}(\text{OH})_2$  reagent, sometimes by means of finely powdered baryta and baryta water [Eggleton and Eggleton, 1929-30; Cori and Cori, 1931-2a].

#### PHOSPHAGEN.

Evidence for the absence of creatinephosphoric acid from the cold extract and for the presence of a substance with a rate of hydrolysis resembling that of argininephosphoric acid was obtained in the following way. The preformed inorganic phosphate in a portion of the neutralized extract was precipitated by the Fiske and Subbarow calcium reagent, the precipitate was dissolved in acid, and the phosphate estimated. A second portion of the extract was allowed to stand 15 min. with the molybdate reagent before the aminonaphtholsulphonic acid was added. This treatment should cause complete breakdown of any creatinephosphoric acid present, but no rise in inorganic P was found. A third portion was diluted with water or partly neutralized so as to be about  $N/20$  in acidity, and then incubated at 28° C. overnight. An increase in inorganic phosphate was then found, and this increase may be regarded as the argininephosphoric

acid P. In amount it varied between 10 and 20 mg. per 100 g. The pre-formed inorganic phosphate in the same experiments varied between 17 and 25 mg. per 100 g.

In other cases the centrifugate after removal of the insoluble calcium salts was used. Here inorganic phosphate was not initially present, but was set free during the incubation period. In order that the base combined with phosphate might be further examined, apart from any free bases present, such a centrifugate was treated with two volumes of ice-cold alcohol; a precipitate consisting of the calcium salt of the phosphagen together with the calcium salt of a hexose ester was obtained, which, after standing, was spun off, washed and then dissolved in normal acid. It was left at room temperature overnight, so that phosphagen present might break down, and then tested:

(a) By Weber's modification [1930] of the Sakaguchi reaction for arginine. This gave a good colour, corresponding roughly to the amount of labile phosphorus which had been found in the extract. No free base was present before incubation.

(b) By Walpole's diacetyl test [1911], which was negative. Controls showed that the calculated amount of creatine (from the labile phosphorus) gave a good pink colour. The calculated amount of arginine gave no colour.

(c) The Jaffé reaction was negative.

We next investigated, by two methods, the effect of arginase (prepared according to Edlbacher and Röthler's directions [1925]) on the base combined in the phosphagen.

(a) *By the Weber-Sakaguchi method.* The precipitate containing the calcium salts of the phosphagen and hexose ester was prepared from about 400 flies, as described above. It was dissolved in decinormal acid and heated in a boiling water bath for 15 min. to hydrolyse the phosphagen. After cooling and neutralizing, two volumes of alcohol were again added; this time the calcium salt of the hexose ester was precipitated, the base, being now free from phosphate, remaining in solution. The calcium precipitate was well washed, and the washings added to the centrifugate.

After removal of the alcohol, the solution was made up to 50 c.c. By the Weber-Sakaguchi reaction it was shown to contain 0.025 mg. arginine per c.c. A standard arginine solution of the same strength was made up. 5 c.c. of each, the standard arginine and the extract, were then incubated at 37° C., each having received one drop of the diluted arginase solution, and a few drops of toluene. The pH was about 9. It is usual to

employ a glycine buffer with arginase to control the  $pH$ , but this had to be omitted in these experiments, since glycine interferes with the Weber-Sakaguchi reagents. After 2 hours, the amount of base present, from the intensity of the colour with the Weber-Sakaguchi reagents, had been reduced to about one-eighth of that present before incubation; the residual coloration was the same in both tubes, and was not reduced by further incubation. This residual colour was due to the presence of proteins in the arginase solution, as was shown by control experiments. After deproteinization of the test solutions by means of trichloroacetic acid, no coloration was produced.

(b) *By the urease method*, in which the production of urea by the action of arginase is shown by the action of urease on this urea. 123 flies (giving 3 g. of thoraces) were extracted with 24 c.c. trichloroacetic acid. The insoluble calcium salts were removed, and to the centrifugate two volumes of alcohol were added as usual. The precipitate was dissolved in a little HCl, made approximately decinormal, and incubated overnight at  $37^{\circ}C$ . to hydrolyse the phosphagen. The solution was then neutralized and made up to 10 c.c., 4.5 c.c. were treated with 2.2 c.c. glycine buffer ( $pH$  9.0) and 0.3 c.c. of diluted arginase solution. The whole was incubated for 3 hours under a layer of toluene at  $37^{\circ}C$ . The solution was then boiled to destroy the enzyme and brought to  $pH$  7, when a few drops of Folin's pyrophosphate buffer were added. 12 mg. of urease (finely powdered) were added, and incubation for 1 hr. at  $37^{\circ}C$ . followed. The ammonia was distilled off by Watchorn and Holmes' modification [1927] of Stanford's method [1923], and estimated, after Nesslerization, in the colorimeter. Blanks were performed on the enzyme solutions, etc., without the extract and on the extract alone. A similar experiment was carried out on an arginine solution (0.005 p.c.) in order to see what yield of ammonia might be expected with these very small quantities of arginine (Table I).

TABLE I.

	mg. $NH_3$ -N found
4.5 c.c. extract without enzymes ... ..	0.011
Arginase, urease, etc., without extract ... ..	0.009
Reagents alone ... ..	0.002
Total blank ... ..	0.022
4.5 c.c. extract + enzymes ... ..	0.088
$\therefore NH_3$ -N from arginine of extract ... ..	0.066
4 c.c. 0.005 p.c. arginine solution without enzymes ... ..	0.004
Arginase, urease, etc., without arginine solution ... ..	0.009
Reagents alone ... ..	0.002
Total blank ... ..	0.015
4 c.c. 0.005 p.c. arginine solution + enzymes ... ..	0.037
$\therefore NH_3$ -N from arginine in solution ... ..	0.022

From 1 c.c. of the same extract it was found that 0.022 mg. free P was present per c.c. This had all arisen by breakdown of argininephosphoric acid, so that the presence of 0.124 mg. arginine, and the production of 0.020 mg.  $\text{NH}_3\text{-N}$  per c.c., were to be expected. 73 p.c. of this amount was actually found. 4 c.c. of the standard should yield 0.032 mg.  $\text{NH}_3\text{-N}$ ; 69 p.c. of this amount was obtained. Thus both the Weber-Sakaguchi method and the arginase-urease method show that the base of the phosphagen is attacked by arginase.

Further evidence for the fact that the base in question is arginine is given by the rate of hydrolysis of the phosphagen in presence of molybdate ions. When muscle extracts were incubated, as usual in *N/10* acid, in the presence of 0.3 p.c. ammonium molybdate (the concentration used in the experiments of Meyerhof [1928]) an unexpected difficulty was encountered in the phosphate estimations, which was traced to the presence of small amounts of silicate in the various reagents. The molybdate appeared to have the effect of greatly increasing the rate of liberation of phosphate, but it was soon observed that the amounts estimated as set free were impossibly high, being in some cases greater than the total P present. The explanation seems to be that silicate unites with molybdic acid in decinormal acid solutions to give silicomolybdate; the yellow colour consequent on this reaction is used as the basis of an estimation method for silicate. The silicomolybdate, we find, can be rapidly reduced in more strongly acid solutions by aminonaphtholsulphonic acid to give a blue compound, thereby simulating phosphoric acid. The formation of silicomolybdate does not take place in more acid solutions and consequently the appearance of spurious phosphate only occurs in the Fiske and Subbarow method if it is used on a solution which has previously been treated with molybdate in the presence of *N/10* acid. Fiske and Subbarow, indeed, showed that the presence of silicate in a phosphate solution may retard the production of colour in their method.

Instead of using some other method of phosphate estimation, we decided to follow the effect of molybdate on hydrolysis rate by means of the arginine set free, using the Weber-Sakaguchi method (which gives no colour with argininephosphoric acid), in the manner described by Baldwin [1933].

Fifty thoraces (which weighed 0.98 g.) were extracted with 5 p.c. trichloroacetic acid. After filtering, the solution was diluted to 20 c.c., and soda was added until thymol blue, used as external indicator, showed the *pH* to be about 1.5. The solution was divided into parts: to 10 c.c., 1 c.c. 3 p.c. ammonium molybdate was added; to another 6 c.c.,



0.6 c.c. water was added. The two solutions were stoppered and incubated at 28° C. Samples of 0.5 c.c. were removed at intervals, and the free arginine was estimated. The method is not so dependable as the Fiske and Subbarow phosphate method, but the results, shown in the accompanying curves (Fig. 1), enabled us to calculate the following velocity constants:

0.3 p.c. molybdate present	$k = 0.58 \times 10^{-3}$
Control ... ..	$k = 7.7 \times 10^{-3}$

( $k$  values are expressed in natural, not decadic logarithms)

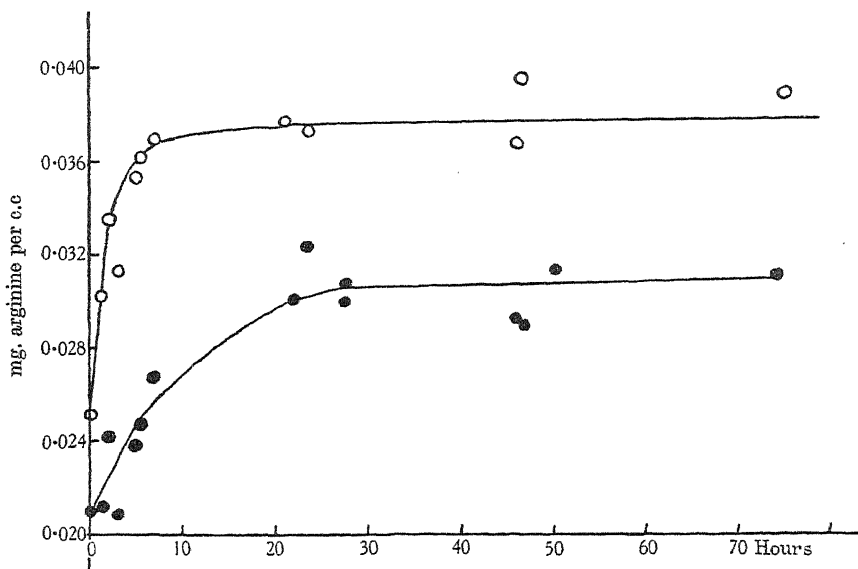


Fig. 1. The effect of molybdate ions on the hydrolysis of phosphagen.  
 ○—○ without molybdate; ●—● with 3 p.c. ammonium molybdate.

There is thus a retardation of 14 times in the presence of the molybdate ion. Meyerhof and Lohmann [1928] and Meyerhof [1928] find that the retardation so produced in crude extracts is of the order of 15, with which our result is in good agreement; our absolute  $k$  values also agree well with those of the German workers.

All the evidence available, therefore, points to the presence of arginine-phosphoric acid in fly muscle. This could only be made certain (*a*) by the preparation and analysis of the pure compound, or (*b*) by the preparation of a crystalline derivative of the base suitable for X-ray analysis. So far, sufficient quantities have not been obtained for either of these methods.

One crystalline derivative, the flavianate, was indeed prepared, but this, owing to the large size of the flavianic acid molecule, is not suitable for distinguishing between arginine and some closely similar compound either by gravimetric or X-ray analysis. Crystalline compounds of arginine in combination with a small molecule, *e.g.* the nitrate, are far more difficult to obtain when only a few mg. of arginine are available.

To obtain the flavianate, 400 flies were used. The insoluble barium salts were removed, and the centrifugate was treated with two volumes of alcohol. After standing in ice for 2 hours, the solution was centrifuged. The residue was well mixed with  $N/10$   $H_2SO_4$ , and the precipitated  $BaSO_4$  was spun off. The centrifugate was incubated at  $28^\circ C.$  overnight. Next day, the *pH* was adjusted again to 9, with baryta, and two volumes of alcohol were again added to remove the calcium salt of the hexose ester. The centrifugate was evaporated to 7 c.c. and 100 mg. of flavianic acid were added. The flavianate came out on standing, first as red balls. These were centrifuged off and recrystallized from boiling  $H_2O$ , when their appearance approximated closely to that of arginine flavianate, prepared in a similar way from pure arginine. Identification by means of the melting point is not possible, as the only datum given by Kossel and Gross [1924] is that, by  $258-260^\circ C.$ , arginine monoflavianate has turned deep brown without melting.

#### ADENYLPYROPHOSPHATE.

When the Fiske and Subbarow  $CaCl_2-Ca(OH)_2$  reagent was added, in the proportions described by these authors, to the neutralized trichloroacetic extract of the fly muscle, a copious white precipitate was obtained. After standing this precipitate was spun off, and washed as recommended by Fiske and Subbarow. This precipitate contains all the preformed inorganic phosphate of the muscle; in the case of the frog such a precipitate contains besides the inorganic phosphate only adenylypyrophosphate [Eggleton and Eggleton, 1929-30]. The pyrophosphate is estimated by means of the increase in free phosphate after 7 min. hydrolysis at  $100^\circ C.$  in  $N$   $HCl$ ; in the frog the difference between the total phosphate and the free phosphate after 7 min. hydrolysis is the adenylic acid phosphorus, and amounts to half the pyrophosphate phosphorus. In the case of the rat, examined by Cori and Cori [1931-2*a*], the composition of the precipitate was found to vary according to the *pH* at which the precipitation took place. The ratio of the readily hydrolysable (pyro) phosphate to the difficultly hydrolysable was never 2 to 1 if the *pH* was higher than 7.4, but always less than 2 to 1. They showed this to be due

to the adsorption of the calcium salt of hexosemonophosphate on the precipitate, the adsorption being greater the higher the *pH*. Washing would not remove the hexosephosphate from the precipitate. It was necessary to dissolve it and reprecipitate to obtain it free from this contamination. The method adopted by the Coris was to precipitate with barium hydroxide at *pH* 8.6; this precipitate was dissolved in acid and again precipitated at 8.6. This difficulty is not encountered with the frog, as the amount of hexosemonophosphate present is negligible—2–3 mg. P/100 g. instead of about 10 as in the rat. With the fly muscle, which, as we shall see, also contains large amounts of hexose ester, we had considerable difficulty. Precipitation with the Fiske and Subbarow reagent, which had been used during the phosphagen estimations, had to be abandoned as the *pH* was too high. Even when the method of the Coris was followed, the ratio of readily hydrolysable to difficultly hydrolysable phosphate was much too low. We found, however, that by precipitating with finely ground baryta at *pH* 7.4–7.8, then dissolving and re-precipitating at this *pH*, a precipitate was obtained in which the ratio of readily hydrolysable phosphorus to difficultly hydrolysable phosphorus was exactly 2 to 1. The disadvantage of this procedure is that the inorganic phosphate is not completely precipitated at this *pH*, so that, to obtain the true preformed inorganic phosphate, a separate estimation is necessary. Table II summarizes some typical results.

TABLE II.

Exp.	<i>pH</i>	Precipitant	<i>I</i>	( <i>I</i> + $\pi$ )	<i>T</i>	$\pi$	$\alpha$	$\pi/\alpha$
34	10	Ca (F. and S. reagent)	31.0	65.7	99.14	34.7	33.44	1.03
36	10	Ca (F. and S. reagent)	38.1	62.8	84.7	24.7	21.9	1.13
37	9	Ba	21.5	35.3	46.8	13.8	11.5	1.20
39	9	Ba	36.8	67.8	90.1	31.0	22.3	1.39
38	8	Ba	44.6	78.1	94.9	33.4	16.8	1.99
43	7.8–8	Ba	27.6	58.8	73.8	31.2	15.0	2.08
44	7.4	Ba	20.0	50.9	67.2	30.9	16.3	1.90
50	7.4	Ba	23.3	65.4	86.4	42.1	21.0	2.00

The results are expressed in mg. P/100 g. thorax.

*I* = inorganic,  $\pi$  = pyro, *T* = total in precipitate,  $\alpha = T - (I + \pi)$ .

We followed the hydrolysis of the adenylypyrophosphate in *N* HCl at 100° C. over many hours, and obtained curves very similar to those of Lohmann [1928 b] (Figs. 2, 3).

In the particular experiment shown in Fig. 2 the precipitate obtained by two precipitations at *pH* 7.4–7.6 from 120 flies was dissolved in the least possible amount of *N*/10 HCl. The solution was diluted and barium

was removed by adding a concentrated sodium sulphate solution till precipitation was just complete and then centrifuging. To the centrifugate 2 c.c. of 10 *N* HCl were added and the volume was made up to 20 c.c. Samples of 1 c.c. were placed in 10 c.c. volumetric flasks, which were weighted so that they could be suspended in a rapidly boiling water bath. One minute after immersion the flasks were securely corked. They were removed at intervals, and each was placed in ice at once. The

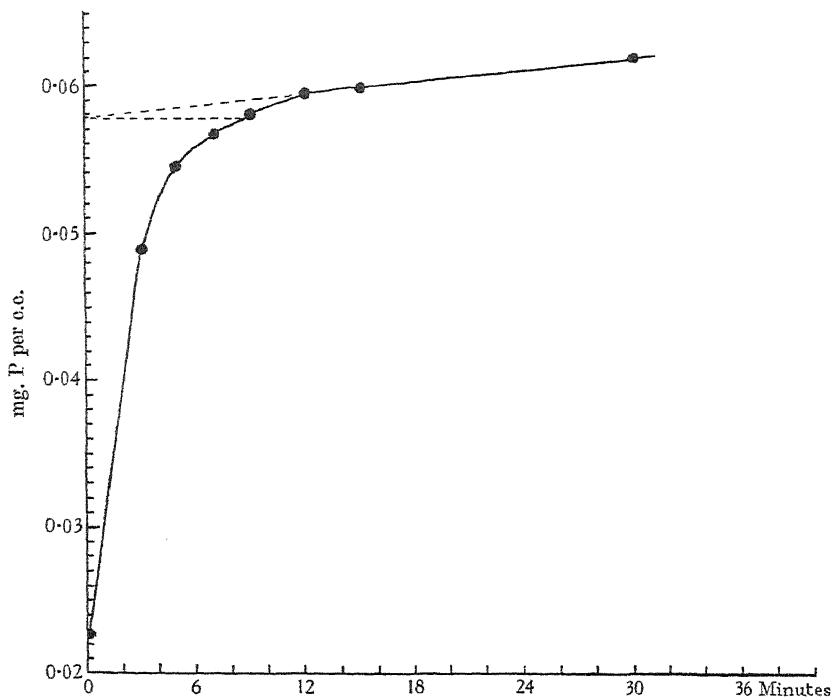


Fig. 2. Exp. 43. Hydrolysis of adenylypyrophosphate in *N* HCl at 100° C.

amount of free phosphate present in each was determined, as well as the initial phosphate and the total phosphate. For the latter the procedure used by Eggleton and Eggleton [1929-30] was followed: 1 c.c. of the extract was heated with 1 c.c. 10 *N* H<sub>2</sub>SO<sub>4</sub> till white fumes appeared, then 2 drops of Merck's perhydrol were added. Gentle heating was continued until the liquid was quite colourless and until all effervescence was ended. After cooling, a little water was added, and the contents of the tube were boiled for 2 min. to decompose any remaining hydrogen peroxide, and any pyrophosphate and persulphate formed during the incineration. In

performing the phosphate estimation, it was necessary to allow for the  $\text{H}_2\text{SO}_4$  added in the incineration, as the presence of excess of acid inhibits colour formation; 2 c.c. of the molybdate solution (2.5 p.c. in 5N  $\text{H}_2\text{SO}_4$ ) were replaced by 2 c.c. of a 2.5 p.c. solution in water.

The value of  $k$  for the pyrophosphate hydrolysis was about  $390 \times 10^{-3}$  reckoned with natural logarithms; reckoned with decadic logarithms this becomes  $170 \times 10^{-3}$ , which may be compared with Lohmann's value of about  $250 \times 10^{-3}$  [1928 *a*]. We usually found that 8–8½ min. heating was necessary for complete hydrolysis of the pyrophosphate, instead of the 7 min. given by Lohmann. For example, in Fig. 2, the phosphate set

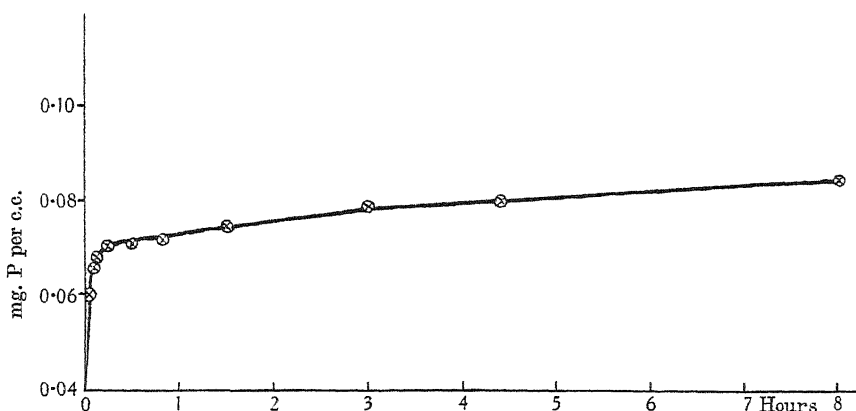


Fig. 3. Hydrolysis of adenylypyrophosphate.

$k$  for the pyrophosphate breakdown =  $171 \times 10^{-3}$ ,

$k$  for the adenylic acid breakdown =  $2 \times 10^{-3}$  (using decadic logarithms).

free in the early stages by breakdown of adenylic acid may be found by extrapolating the steady slope of the curve backwards to the ordinate, and calling the phosphate value, where it cuts the ordinate, zero. If the phosphate values from this curve are subtracted from the experimental values, it is found that the pyrophosphate breakdown is complete in 8½ min. With the precipitates contaminated with hexose ester, this slowing of the pyrophosphate hydrolysis was particularly marked. In agreement with the results of Cori and Cori [1931–2*a*] with rat adenylypyrophosphate, it was found that, in the case of the fly, two-thirds of the adenylic acid phosphate was set free in 4 hours; the value of  $k$ , about  $2 \times 10^{-3}$ , is the same as the value calculated by Myrbäck and Euler [1931] from Levene and Jorpes' data [1929] for inosinic acid hydrolysis (using decadic logarithms).

As is well known, if the attempt is made to estimate the ribose in mammalian adenylypyrophosphate by the Hoffmann distillation method [1927], the yield of furfural is only a small proportion of that to be expected. Thus Embden and Schmidt [1929] report the production of only traces of furfural from rabbit adenylic acid; Steudel and Wohinz [1931] obtained 12 p.c. of the expected amount using inosinic acid from meat extract; Barrenscheen and Filz [1932] found 6 p.c. of the expected furfural was produced on distillation of rabbit adenylypyrophosphate. In our own experiments on fly muscle we obtained a slightly higher yield, but as a similar yield was obtained from frog adenylypyrophosphate, this fact is probably not of significance for the structure of the adenylic acid. In our experiments the procedure of Hoffmann was followed exactly, including the use of distillation apparatus with mercury-sealed joints, so that the use of rubber was avoided.

Some results are shown in Table III.

TABLE III.

Exp.	Material	Wt. used	Furfural expected mg.	Furfural obtained mg.	Yield %
28	Yeast adenylic acid	37.3 mg.	5.2	4.35	84
28	Furfural	5 mg.	5.0	3.85	77
28	Frog muscle	Ca ppt. from 5.3 g.	2.1	0.58	27.5
29	Frog muscle	Ca ppt. from 12 g.	3.75	1.38	37
20	Fly muscle	Ca ppt. from 6 g.	1.816	0.454	25
47	Fly muscle	Ba ppt. from 3.4 g.	1.36	0.35	25
50	Fly muscle	Ba ppt. from 4.6 g.	2.98	0.89	30
	Muscle adenylic acid	10.54 mg.	2.9	0.5	16

The expected furfural was calculated as the equivalent of half the pyrophosphate P, estimated in a sample of each muscle extract.

It was desired to compare, if possible, the result obtained for the adenylic acid of the muscle extracts by phosphate estimations with the result obtained by pentose estimations. It is obviously impossible to estimate the pentose content of muscle adenylic acid by Hoffmann's method; the method of Ferdmann [1933], in which 70 p.c. yields of furfural can be obtained from muscle adenylypyrophosphate by distillation in 6*N* H<sub>2</sub>SO<sub>4</sub> at 150°C., was not published till after this work was completed. An attempt was therefore made to estimate the pentose by means of the orcein-ferric chloride reaction of Bial. The estimation was carried out as described by Scheff [1924] for pentoses, except that a colorimeter was used instead of a spectrophotometer. The barium precipitate was dissolved in acid, and the volume was adjusted so that 1 c.c. should contain about 0.20 mg. adenylic acid (calculated from the figures found for pyrophosphate). 1 c.c. was placed in a dry tube and treated

with 4 c.c. of the Bial reagent. After 1 min. heating in a boiling water bath the tube was firmly stoppered (with a cork covered in tinfoil) and the heating was continued for another 9 min. After cooling, the solution was extracted by shaking, until completely colourless, with 5 c.c. amyl alcohol. After 10 min. the amyl alcohol layer was pipetted off and compared with a similar solution, prepared from muscle adenylic acid and containing 0.2 mg. adenylic acid per c.c. We are indebted to Dr Drury and to Dr P. Ostern for samples of muscle adenylic acid, in both cases made from mammalian muscle. The results obtained were unsatisfactory, being rather variable and always higher than was to be expected from the pyrophosphate results (Table IV).

TABLE IV.

Adenylic acid P (half pyro P) mg. per c.c. extract	Adenylic acid P (from pentose) mg. per c.c. extract
0.0170	0.0201
0.0170	0.0186
0.0170	0.0251
0.0177	0.0205
0.0177	0.0207

An attempt was also made to use the modification of Sumner [1923], as Dr Cori informed us in a private communication that this was the method successfully used in the work on the rat. With the fly preparations, however, this method also gave high results, and the colour produced was not the same in the unknown as in the standard. Further work must show whether these results are due merely to the presence of some factor in the fly muscle influencing the rate of furfural production, or whether they are due to some peculiar property of fly adenylic acid. It may be mentioned that with Scheff's modification of the Bial reaction, colour production was always markedly more rapid with the standard muscle adenylic acid solutions than with yeast adenylic acid solutions of the same concentration; after the 10 min. heating the colour developed in the yeast adenylic acid tubes was only about 75 p.c. of that in the muscle adenylic acid tubes.

#### THE HEXOSE ESTER FRACTION.

The total P content of the trichloroacetic acid extract was shown to be considerably greater than the sum of the inorganic, phosphagen and adenylypyrophosphate P. As we shall see, this P probably belongs to hexosemonophosphate. The excess of P amounted, in two of the earlier

experiments, to 14.3 mg/100 g. and 16.4 mg./100 g. These values are probably rather low, as in these particular experiments precipitation had been carried out at pH 10, so that the values for adenylypyrophosphate subtracted from the total were too high. After precipitation at pH 7.4, the value of 19.4 mg./100 g. was found. In some later experiments the method of Cori and Cori [1931-2*a*] for the estimation of hexosemonophosphate (with one or two adaptations necessitated by the different material) was followed, and here again, as was to be expected, higher results were obtained.

In one such experiment the extract from 84 flies was neutralized to pH 7.4-7.6 with baryta. To the centrifugate after removal of the precipitate, four volumes of 97 p.c. alcohol (neutralized) were added, and the mixture was kept overnight at 0° C. In this way argininephosphoric acid and hexosemonophosphate are completely precipitated. Then the precipitate was centrifuged off and dissolved in 20 c.c. *N*/10 HCl; the barium was removed by addition of sodium sulphate solution followed by centrifuging. The argininephosphoric acid was then broken down by 15 min. heating in a boiling water bath. The free phosphate present after this treatment was estimated in one sample (*A*), as well as the total P in another sample (*B*).

The values obtained were:

<i>A</i>	...	...	25.7 mg. free P/100 g.
<i>B</i>	...	...	48.0 mg. " "
Ester P	...	...	22.3 mg. " "

The value *A* represents, of course, the phosphagen P, together with some preformed inorganic P which escaped precipitation by barium, on account of the low pH.

In another experiment the reducing value of the ester was compared with the P content as was done by the Coris. Three hundred flies were used; the precipitation was again at pH 7.4; in order to avoid any reduction by arginine, the argininephosphoric acid was hydrolysed (in *N*/10 acid for 30 min. at 100° C.) before the alcohol precipitation. After the hydrolysis, the solution was neutralized, and four volumes of neutralized alcohol were added. After standing for 3 hours the precipitate was centrifuged off; it was dissolved in acid, and the barium was removed by sodium sulphate solution. The volume was made up to 15 c.c. The free phosphate (*A*) present in 1 c.c. was estimated, and the total P (*B*) in another 1 c.c. sample. Two samples of 5 c.c. each were used for the estimations of reducing power by Hagedorn and Jensen's method, using the solutions described by Boyland [1928].



The phosphate estimations gave the following results:

A	...	...	24.9 mg. P/100 g.
B	...	...	43.5 "
Ester P	...	...	18.6 = 108 mg. hexose/100 g.

The reducing power of 5 c.c. was equivalent to 1.57 mg. glucose. For 100 g. the value would be 82.6 mg. As the reducing power of hexose-monophosphate is only 67 p.c. of that of glucose, the value must be multiplied by 100/67, in order that it may be compared with the hexose calculated from the P value. The result, 123 mg. hexose/100 g., is 14 p.c. high. This point will be further investigated, but at present it seems more reasonable to conclude that the presence of small amounts of other reducing substances (*e.g.* glutathione) [see Cori and Cori, 1931-2 *a*] is responsible rather than that a different hexosephosphate is present.

#### DISCUSSION.

It will be interesting to compare the general distribution of soluble phosphorus found in the fly with the results given by Eggleton [see Hill and Kupalov, 1930] for the frog and by Cori and Cori [1931-2 *a*] for the rat.

TABLE V.

	P mg./100 g. muscle		
	Fly	Frog	Rat
Orthophosphate ...	25-35	18	} 115
Phosphagen ...	10-20	65	
Pyrophosphate ...	30-40	30	
Esters with soluble Ba salts ...	20	5	7-10
Esters with insoluble Ba salts (adenylic acid of adenylypyrophosphate) ...	15-20	15	20.5

One striking difference is the low phosphagen content of the fly muscle. It is also considerably lower than that found in many of the other invertebrates, especially other insects by Schütze. It is, of course, possible that the value found after the treatment described is lower than that *in vivo*; that the argininephosphate P could not, however, even *in vivo* be higher than about 20 mg./100 g. is shown by experiments in which the total free arginine was estimated after hydrolysis of the phosphagen. The value obtained for the free arginine in such an experiment was 121 mg./100 g. the equivalent of which in P is only 21.5 mg./100 g.

Secondly, the fly muscle shows a high content of hexose ester, compared with the frog muscle, and with rat muscle removed under amytal anaesthesia. As Cori and Cori [1932-3] have pointed out, stimulation of excised rat muscles to contraction leads to increase in hexosemono-

phosphate content, but this increase soon disappears if the muscles return to rest. The low resting value of hexosemonophosphate P, 7–10 mg./100 g., was only obtained when the muscle was removed from the animal under amytal anaesthesia. The nervous discharges accompanying death by stunning, decapitation, etc., caused great rise in hexosemonophosphate content. Injection of epinephrine into the intact animal also led to the production of hexosemonophosphate in the muscles, and the rise might persist for some hours [Cori and Cori, 1931–2 b]. Possibly the cold to which the intact flies are subjected acts as a stimulus to their nervous system, and the result is the production of some epinephrine-like substance. In the rat the phosphate necessary comes apparently from the free phosphate of the muscles themselves and of the blood. Both in their low phosphagen content and in their high ester content the fly muscles resemble the muscle of the frog's heart. The following values are from the paper by Clark, Eggleton and Eggleton [1931].

	mg. P/100 g.
Inorganic ... ..	11.6
Phosphagen ... ..	7.3
Pyrophosphate ... ..	11.2
Organic phosphates with insoluble Ba salts	11.3
Organic phosphates with soluble Ba salts	43.1

In the case of these intact, isolated, cold-blooded hearts there is no reason to suppose that the low phosphagen content and the high ester content found *in vitro* do not represent the state of affairs *in vivo*. Further work must decide the question for the fly.

#### SUMMARY.

1. It has been shown that fly muscle contains argininephosphoric acid.
2. The distribution of the acid-soluble phosphorus has been quantitatively studied. The muscle contains an adenylypyrophosphate, probably the same as that in frog muscle, and a reducing ester, probably hexosemonophosphate.
3. The concentration of adenylypyrophosphate is very similar to that in the frog or rat; the argininephosphoric acid content is small and the hexose ester content high in comparison with the frog or rat.

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THE EXCRETION OF NON-METABOLIZED SUGARS  
BY THE MAMMALIAN KIDNEY.

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THE search for renal function tests, which shall be based on sound physiological principles and not solely on empirical observation, has of recent years been conducted in two main directions. Firstly by attempts to form a true estimate of the volume of glomerular filtrate, and secondly by the study of phenomena which are presumably based on the activity of the renal tubule cells.

It is clear that once the true volume of glomerular filtrate is known, the rôle of the tubule cells will immediately be more evident, and it is accordingly of great importance that any claims to have found a method of measuring glomerular filtration rate should be critically examined.

Evidence continues to accumulate, from the work especially of Richards and his associates [1933], that the composition of the filtrate is the same as that of an ultrafiltrate of plasma. Hence, if any substance can be found which enters the tubule lumen by filtration through the glomerulus, and in its passage down is neither added to by secretion or diffusion through the tubule cells, nor reduced in amount by reabsorption in these cells, then that substance can be employed to indicate the volume of glomerular filtrate. The "clearance" for this substance must be identical with the volume of glomerular filtrate [Cope, 1931].

After the abandonment of urea for this purpose by Cushny, there remained two substances which, it was felt, might still fulfil the required conditions. These two were creatinine and inorganic sulphate. But since it was shown that these substances had entirely different clearance values in the human kidney, that of the former being about three times as great as that of the latter, it was evident that one at least of them would have to be discarded [Cope, 1932; Hayman and Johnston, 1932].

Rehberg and the Scandinavian workers generally have persistently upheld their claims for creatinine, although the evidence they bring in support is relatively slender. Hayman and Johnston [1932] attributed the wide discrepancy between the sulphate and creatinine clearances to a tubular reabsorption of the former rather than to any secretion of the latter. Cope [1932], however, felt that the available evidence was entirely inadequate to decide between the apparently rival claims of these substances.

In 1932, however, new substances for consideration were introduced by Jolliffe, Shannon and Smith, *i.e.* the non-metabolized sugars xylose and sucrose. The evidence brought forward in favour of these sugars was much stronger than that which had been accumulated for creatinine.

The main points may be briefly summarized as follows:

(1) Xylose and sucrose have the same clearance value when this is determined simultaneously for both in the mammalian kidney [Jolliffe, Shannon and Smith, 1932].

(2) Under the action of phlorrhizin glucose excretion increases until the glucose clearance comes to equal that already shown by xylose and sucrose [Jolliffe, Shannon and Smith, 1932].

(3) Xylose is readily excreted by fish whose kidneys possess glomeruli, but only in traces by those with aglomerular kidneys [Jolliffe, 1930; Clarke and Smith, 1932].

(4) Glucose is excreted by glomerular but not by aglomerular kidneys in fish under the action of phlorrhizin [Marshall and Grafflin, 1928; Marshall, 1930].

(5) After phlorrhizin the glucose clearance comes to equal the xylose clearance in fish with glomerular kidneys [Jolliffe, 1930; Clarke and Smith, 1932].

(6) Similar phenomena are also presented by the frog's kidney [Marshall, 1932].

These observations are readily explained only if it is supposed that all three sugars are normally filtered through the glomeruli and do not enter the tubule lumen through the tubule cells except in traces, and that normally glucose is reabsorbed by an activity of the tubule cells which can be inhibited by administration of an adequate dose of phlorrhizin.

If this be admitted, then certain further results of these workers must be held to show that creatinine is excreted in part at least by the tubule cells. For Shannon, Jolliffe and Smith [1932] found the normal creatinine clearance in dogs to be 15-50 p.c. higher than that for xylose

(or sucrose), while they claimed that after phlorrhizin the creatinine clearance fell to the same level as that for xylose, sucrose and glucose. And Clarke and Smith [1932] showed further that in the dogfish also creatinine is excreted more readily than xylose, a fact which they interpret as evidence of tubular secretion of the former.

In this respect also, certain recent experiments by Höber [1932] are of interest. He found that when creatinine was supplied to the glomeruli of the frog's kidney by perfusion into the aorta, it was readily excreted. But when it was supplied by the portal vein to the tubules it was also excreted though in greatly diminished amount, a fact which he considers as evidence that, although most of the creatinine is eliminated by glomerular filtration, some leaks through the tubule cells.

It is clear, therefore, that the work of Smith and his associates is of such importance as to require early confirmation.

White and Monaghan [1933] attempted to repeat the observations on the behaviour of creatinine and glucose during their excretion under the action of phlorrhizin, but found that in only one experiment out of thirteen did the glucose clearance rise to equal the creatinine clearance.

The present work also was undertaken with a view to determining to what extent the results of Smith and his co-workers could be confirmed.

Unfortunately facilities were not available for the use of dogs, and accordingly rabbits had to be employed, so that it is not possible to compare strictly the results here presented with the findings of Smith and his associates. Those that agree may be regarded as confirmatory, but those that differ may possibly be due to the use of a different experimental animal.

#### METHODS.

*Creatinine* in urine and plasma has been estimated by the standard methods with alkaline picrate, taking the usual precautions as to purity of picric acid and similarity of concentration of standard and unknown solutions. Plasma concentrations were always determined before the corresponding urine values, and were throughout done on 1 in 10 Folin-Wu filtrates. In the earlier experiments an ordinary Duboscq colorimeter was employed, but in the later a Leitz universal compensating colorimeter was used. With this instrument the insertion of the green light filter greatly increased the convenience and accuracy of the colour comparison.

*Sucrose in plasma.* The principles of the methods used by Jolliffe, Shannon and Smith [1932] have been employed as regards incubation with sucrase at 40° C. for 1 hour at pH 4.0-4.5 (to bromphenol blue) and

subsequent neutralization with sodium hydroxide (to phenolphthalein). For the actual sugar estimation the titrimetric micro-method of Shaffer-Hartmann as modified by Somogyi [1926] was considered preferable to a colorimetric method, and recoveries for both glucose and sucrose were within 2 p.c. of theoretical in known solutions. Duplicate analyses made in all cases were in good agreement.

Sucrose values were obtained by subtracting the plasma glucose figures from those for glucose + sucrose after inversion with sucrase. When only sucrose figures were required Folin-Wu filtrates were used, but when other sugars were also being determined the copper filtrates of Somogyi [1931] were used. Early difficulties in preparing clear filtrates from rabbits' plasma by this method were found to be due to the fact that more copper sulphate was required than for human blood to bring the final pH of the filtrate to about 6.0. In the proportions of 1.5 c.c. of 5 p.c. copper sulphate and 1.0 c.c. of 6 p.c. sodium tungstate to 1.0 c.c. plasma and 6.5 c.c. of water, satisfactory filtrates were obtained.

*Sucrose in urine* was determined by essentially the same procedure using suitable dilutions of the urine, usually 1 in 100 or 200. Somogyi copper filtrates were usually employed, and invariably so when other sugars were also being determined. When sucrose alone was present the blank values obtained before inversion were practically negligible except when creatinine concentrations were high, for copper precipitation only removed about 60 p.c. of the creatinine in trial experiments. Using the Shaffer-Hartmann-Somogyi micro-method a 1 p.c. solution of creatinine in urine gave a reduction equivalent to about 300 mg. of glucose, and after the precipitation this was reduced to 130 mg. But by using the same analytical method for plasma and urine and diluting the urine to a concentration similar to that of the plasma, the resultant small error was practically eliminated, as Shannon, Jolliffe and Smith [1932] have pointed out. The recoveries of sucrose in urine were within 2 per cent. with satisfactory agreement of duplicates.

*Sucrase* was made by the method of Morrow [1927], after submitting the yeast to a preliminary "enreicherung" before autolysis according to the recommendation of Willstätter, Lowry and Schneider [1925]. 0.2 c.c. of the resultant clear solution was ample for a single analysis, and the blank due to the enzyme solution was small.

*Glucose* in both plasma and urine was always determined from the difference in reducing activity before and after fermentation with washed yeast of copper filtrates according to the procedure of van Slyke and Hawkins [1929].

*Xylose.* For a generous supply of xylose we are indebted to the United States Chamber of Commerce, who also supplied full details of the methods to be used for its purification. It was estimated in both plasma and urine as unfermentable reducing substance in copper filtrates. The finding of Jolliffe, Shannon and Smith [1932] that, during the fermentation, yeast absorbs about 13 p.c. of the xylose, has been confirmed for filtrates containing about 10 mg./100 g. of the sugar. With our yeast it has tended to increase with higher concentrations, reaching 16 p.c. with 50 mg./100 g., and falling to about 10 p.c. in solutions with 5 mg. representing filtrates of a blood with 50 mg./100 g. To avoid complications, however, the observed non-fermentable reductions have been multiplied by 100/87 to obtain the true xylose figures. The Shaffer-Hartmann-Somogyi micro-method with 15 min. heating gives a reduction corresponding to only 95 p.c. of that shown by the same concentration of glucose. But since this factor enters equally into all analyses in which xylose is present and does not affect the calculation of the clearance, it has not been applied. The phlorrhizin used in some experiments must affect the reduction figures. With the sugar method used 100 g. phlorrhizin gave a reduction equivalent to 13 mg. glucose. Since this is apparently not removed either by the copper precipitation or the yeast treatment, it will be estimated in the xylose fraction. But since xylose is present in amount three to four times that of phlorrhizin, the reduction due to the latter will not exceed about 4 p.c. of the former. Hence, unless the two substances have widely different clearances, the phlorrhizin will not greatly affect the estimation of the xylose clearance. Nevertheless, it is probable that the xylose analyses are rather less accurate than those of the other sugars.

#### EXPERIMENTAL.

Male rabbits were used throughout. 40–100 c.c. of an aqueous solution containing such quantities of sucrose, xylose and creatinine as would give approximately the desired plasma concentrations were injected subcutaneously. About an hour was allowed for absorption before collection of urine samples was begun. All urine collections were made by a soft rubber catheter, and although the bladder was not washed out, it is not believed that the volume of residual urine was large. Blood samples were collected at the middle of each urine period from an ear vein, being allowed to drip directly into a centrifuge tube containing potassium oxalate. The samples were centrifuged immediately, and from the plasma



Folin-Wu or copper filtrates were prepared within 2-3 hours. These filtrates, when not analysed the same day, kept well in the cold store overnight.

In no experiment was any anaesthetic used, the animals remaining placid and normal throughout.

When phlorrhizin was administered, it was given by subcutaneous injection dissolved in 1.5 p.c. sodium bicarbonate in a uniform dose of 1 g. to rabbits averaging 2.5-3.0 kg. in weight.

### RESULTS AND DISCUSSION.

A comparison of the sucrose and creatinine clearances was first entered upon before the work of Shannon, Jolliffe and Smith [1932] was published. Sucrose was used in preference to xylose because these workers had previously claimed that the two sugars behaved similarly and were presumably interchangeable. Sucrose analyses were apparently rather more accurate than those with xylose, and further, at the time this work was done, an adequate supply of xylose was not available.

It soon became apparent that the creatinine clearance is practically always somewhat higher than that for sucrose during the same period. In this we therefore fully confirm the published results of the above workers.

In this series a wide range of clearance values was encountered, owing mainly to the fact that two of the rabbits were fed for some weeks on a diet of bran and oats, which was associated with a serious fall in the clearances, a fall which did not occur on a diet of green vegetables.

In Fig. 1 the observed creatinine clearances are plotted against the corresponding sucrose clearance figures. It will be seen that there is a definite proportionality between the two, although more scattering exists than is to be accounted for by experimental errors. The dotted line in the figure is the line of equality of the two clearances. In all cases but one the points lie above this line, the creatinine clearances therefore exceeding the sucrose clearances.

Now the creatinine clearance is the basis of Rehberg's test for renal function, and is extensively used for clinical purposes in Scandinavia and to a less extent elsewhere. It was accordingly felt that, if this test is to justify its use for scientific purposes, it should be feasible to use it to examine the possibility that, in the excretion of substances of this type, there exists an essentially linear relationship between plasma concentration and rate of excretion, but that this is frequently obscured by

spontaneous and irregular variations in general renal activity. A case has indeed been recorded of one human subject in whom, under certain conditions, such a relation did exist for creatinine [Cope, 1931], but this does not appear to be the general experience.

We have therefore used the creatinine clearance as a measure of general renal activity whilst studying the excretion of sucrose at varying plasma concentrations. In such work it is unnecessary to assume the correctness of Rehberg's contention that creatinine measures the glomerular filtrate, and it is for this reason that we prefer to use the term

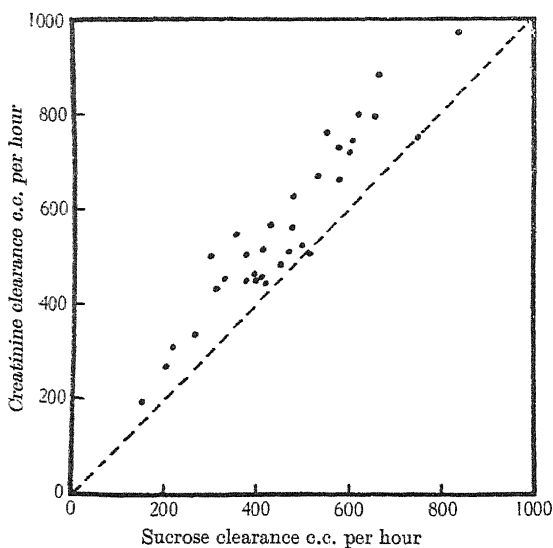


Fig. 1.

general renal activity. In this group of experiments plasma creatinine concentrations have been kept within the relatively narrow limits of 7-14 mg./100 g. in order as far as possible to minimize any possible influence of varying creatinine concentration. Sucrose concentrations have meanwhile been varied from 20-220 mg./100 g. in the plasma. The observed sucrose excretions have been corrected for any variations in this general renal activity as judged by the creatinine clearance, assuming that full activity is indicated by a creatinine clearance of 700 c.c. per hour. Thus, if the observed creatinine clearance in a given period is only 600, the observed sucrose excretion during this period has been multiplied by 7/6, since the kidney was then only exerting 6/7 of its full activity.

In Fig. 2, then, the plasma sucrose concentration has been plotted against the sucrose excretion rate corrected to a constant general renal activity corresponding to a creatinine clearance of 700 c.c. per hour. The suspected linear relation is at once revealed.

If now the conditions are reversed, and plasma creatinine concentration is varied over a wider range, whilst the sucrose clearance is used as an index of general renal activity, the observed creatinine excretion rate can similarly be corrected for variations in this activity in like manner. In Fig. 3 plasma creatinine concentration has been plotted against the observed excretion rate after this has been corrected to a constant renal activity corresponding to a sucrose clearance of 500 c.c. per hour. With

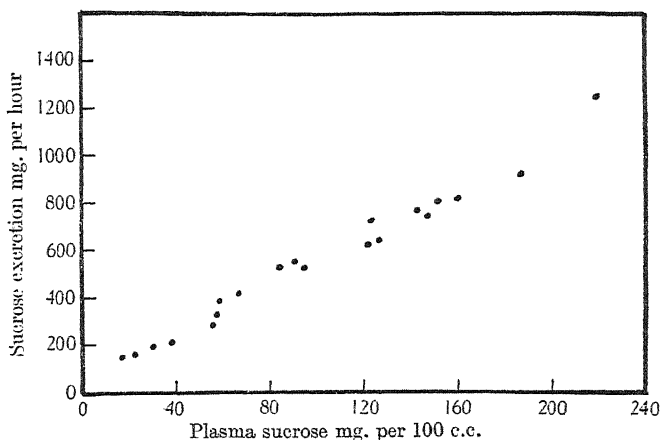


Fig. 2.

the exception of one, all points fall close to a straight line and a linear relationship is again demonstrated. This figure is of interest also from another point of view. The objection is frequently raised against experiments with creatinine that the existence of this substance in normal plasma is in doubt, and that therefore the total chromogenic substance is no measure of the amount of available creatinine. If this were an appreciable factor in determining the excretion of creatinine we should expect its effect to be increasingly evident as the amount of exogenous or added creatinine in the plasma is diminished. Fig. 3 shows, however, that this is not the case. Creatinine excretion has been found to be no less regular when the "apparent" creatinine in plasma is low than when it is high. Yet at the low concentrations the excreted creatinine is presumably derived mainly from the unknown plasma chromogen.

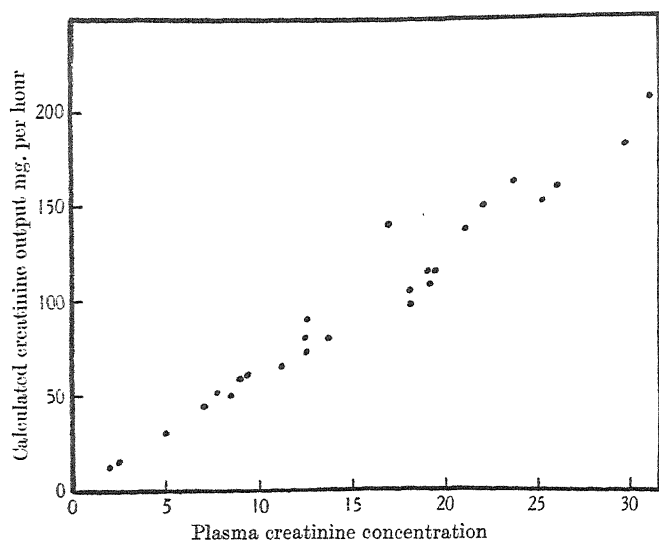


Fig. 3.

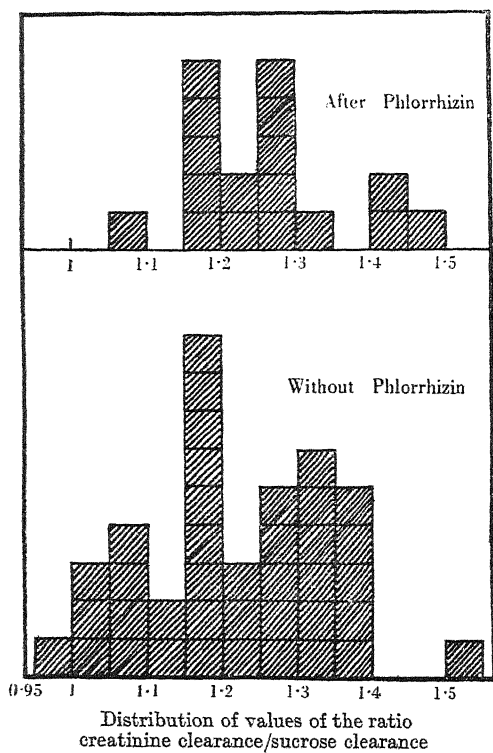


Fig. 4.

This fact, and also the experiments previously reported on a human subject [Cope, 1931], show that in practice no significant error is introduced in work of this kind by assuming that, so far as the kidney is concerned, practically all the available chromogen is treated as creatinine. Whilst we are willing to accept the view that the chromogen of normal plasma does not behave chemically like creatinine, we feel nevertheless that when the plasma concentration is artificially raised, no significant errors are introduced by regarding the whole as creatinine. White and Monaghan [1933] have taken as the creatinine content of plasma in their experiments the observed value minus the value obtained on a normal plasma before administration of creatinine. This method is only permissible if, at the same time, the urine is dealt with in a similar way. That portion of the urinary creatinine which is derived from the normal plasma chromogen must also clearly be subtracted from the total urinary creatinine. If this is not done, the resultant figures for the creatinine clearance will tend to rise to infinity as the proportion of exogenous plasma creatinine falls to zero. Whether or not the urine was so dealt with is not clear from the published figures of the results of White and Monaghan.

We have already noted the fact that in Fig. 1 the deviations from a linear relation between sucrose clearance and creatinine clearance are greater than can be attributed to experimental errors. The extent of variation of the value of the ratio creatinine clearance/sucrose clearance is an indication of this (Fig. 4), for if both linear relations held accurately the ratio would remain constant.

Now Smith and his co-workers explain the fact that the creatinine clearance exceeds that for sucrose by supposing a secretion by the tubules of that portion of the excreted creatinine responsible for the difference between the two clearances. But since this explanation, though a probability, is not yet a certainty, we prefer to allude to this extra creatinine by the less committal term "excess creatinine clearance."

If we suppose that the excretion of this extra creatinine is brought about by a process in the kidney different from that responsible for the excretion of the sucrose on the one hand, and an amount of creatinine corresponding to the same clearance as the sucrose clearance on the other, then it is evident that this process is of very variable activity and may be responsible for from 0 to 30 p.c. or more of the total quantity of creatinine excreted.

Attempts to correlate the variations in the excretion of this fraction, the "excess creatinine clearance," with other factors which might be

expected to influence it, if it be a tubular secretion or diffusion, have not been successful. It apparently bears no relation to the absolute value of the clearance. Nor do the actual concentrations of creatinine in the plasma or the urine seem to be determining factors. This lack of correlation with the plasma concentration is also evidence that the variations in this "excess creatinine clearance" are not attributable to discrepancies between the "apparent" and the "true" creatinine of these plasmas.

The possibility that the variations are rather due to changes in sucrose excretion cannot be ignored, but here again no influence of the concentration of sucrose in plasma or in urine could be detected.

Attempts to influence the value of the "excess creatinine clearance" by urea have also failed, no definite effect having been noted.

We therefore have no real clue as to the origin of this "excess creatinine" or to the reasons for its variations. In their very irregularity they are perhaps most in keeping with the view that this process is one of secretion, and we feel inclined provisionally to accept this explanation in the absence of further evidence.

Now Shannon, Jolliffe and Smith [1932] have claimed that under the influence of phlorrhizin the creatinine clearance falls and becomes equal to the xylose clearance, so that this "excess creatinine clearance" disappears. In our experiments using sucrose in place of xylose it has not been possible to confirm their findings. The values of the ratio creatinine clearance/sucrose clearance obtained in a number of such comparisons are shown in Fig. 4. It will be seen that the distribution is not significantly different from that obtained in the absence of phlorrhizin. There has appeared no constant tendency for the value of this ratio to approach unity, although in most cases the glucose excretion indicated a complete phlorrhization. Typical figures obtained are shown in Table I.

If, however, the creatinine clearance is compared with the xylose clearance a definite fall in the ratio between the two can always be noted. This discrepancy can be accounted for by the fact that in our experiments the xylose clearance has been found to be consistently below that for sucrose in the absence of phlorrhizin, the value of the ratio sucrose clearance/xylose clearance varying between 1.17 and 1.39, with a mean of 1.30. Phlorrhizin has brought this ratio down to unity within the limits of experimental error, the mean value of the ratio then becoming 0.98. We are not prepared to say definitely whether this is due to a fall in the sucrose clearance or to a rise in that of xylose, but are inclined to the view that the sucrose clearance falls to the xylose level.

TABLE I.

Exp.	Urine vol. c.c. per hr.	Sucrose			Xylose			Glucose			Creatinine			Ratios		
		Plasma	Urine	Clear- ance	Plasma	Urine	Clear- ance	Plasma	Urine	Clear- ance	Plasma	Urine	Clear- ance	Cr. Su.	Glu. Su.	Cr. Xy.
1	10-3	193.6	4179	222	—	—	—	165.2	—	—	21.9	656	308	1.39	—	—
	11.8	203.6	3800	221	—	—	—	182.2	2440	158	23.2	510	260	1.18	0.71	—
	10.0	180.4	3283	182	—	—	—	192.4	3605	187	23.6	454	192	1.06	1.03	—
2	7.06	250.0	9403	266	—	—	—	194.9	—	—	29.7	1410	335	1.26	—	—
	7.78	139.5	4085	227	—	—	—	198.6	5428	212	17.2	622	281	1.24	0.94	—
	5.16	100.6	3621	186	—	—	—	209.4	5915	146	14.2	610	221	1.19	0.78	—
3	5.03	76.3	3602	238	—	—	—	156.1	5678	183	11.8	705	300	1.26	0.77	—
	18.5	146.1	3248	411	178.5	3055	316	117.8	—	—	32.0	975	504	1.37	—	1.78
	14.7	83.7	2088	368	79.8	1986	367	125.2	2684	315	21.0	614	430	1.17	0.86	1.17
4	14.1	63.8	1715	380	56.3	1590	399	127.3	3121	346	16.6	645	547	1.44	0.91	1.37
	35.4	118.4	1736	519	150.2	1640	372	128.3	—	—	29.7	505	602	1.16	—	1.02
	18.8	114.4	2195	360	114.0	2300	379	115.5	2164	353	23.9	615	484	1.34	0.98	1.38
5	15.7	70.7	2186	486	73.5	2100	450	121.3	2503	324	17.9	710	622	1.28	0.66	1.38
	25.0	116.0	1814	392	137.0	1690	308	134.5	—	—	17.0	296	435	1.11	—	1.41
	14.8	93.6	2466	391	85.6	2380	412	118.3	3165	396	13.2	429	480	1.23	1.01	1.16
6	10.6	58.9	2327	419	56.2	2040	385	125.7	3045	257	9.7	450	492	1.18	0.61	1.28
	24.0	100.2	2200	526	102.5	1905	446	99.0	—	—	12.2	278	546	1.04	—	1.22
	13.4	47.6	1796	505	44.5	1810	545	103.5	3769	489	6.2	290	628	1.24	0.97	1.15
11.1	28.7	1282	496	510	137.4	1374	510	113.3	5147	503	5.0	287	638	1.28	1.01	1.03
	11.1	28.7	1282	496	137.4	1374	510	113.3	5147	503	5.0	287	638	1.28	1.01	1.03

*Note.* A dose of 1 g. of phlorrhizin was administered after period 1 in each experiment. Period 2 began about 1 hour after this injection, except in Exp. 1 in which it began immediately after the injection.

Nevertheless, the creatinine clearance has never fallen to the xylose level even during the periods when phlorrhizin action was complete as judged by the glucose excretion, the mean value of the ratio creatinine clearance/xylose clearance being then 1.21 compared with 1.55 in the absence of phlorrhizin. In this our results thus agree better with those of Poulsson [1930], and of White and Monaghan [1933] than with those of Shannon, Jolliffe and Smith [1932].

That during the action of phlorrhizin the glucose clearance rises to equal the sucrose and xylose figures we have been able fully to confirm. The mean of the maximum values of the ratio glucose clearance/sucrose clearance reached in each of seven experiments was 0.97.

We have, in these experiments, been dealing with plasma creatinine concentrations which are on the whole considerably lower than those used by Shannon, Jolliffe and Smith. The possibility that herein lay the cause of the failure to reduce the creatinine clearance to the xylose and sucrose level could not be ignored. It seems unlikely that this is the case however. These workers have observed their effect with plasma creatinine concentrations below 20 mg./100 c.c. Moreover, they state that in dogs the non-creatinine chromogenic substance never exceeded 0.5 mg./100 g., so that with plasma creatinine level above 10 mg./100 g. the resultant error is small. The discrepancy we find between the creatinine and xylose clearances under phlorrhizin averages about 20 p.c. and would appear therefore to be much too large to be accounted for in such a way.

We find ourselves quite unable to account for these phenomena of the excretion of the sugars except along the lines of the views held by Smith and his associates. That is, that phlorrhizin diminishes or abolishes the permeability of the tubule cells, so that on the one hand the reabsorption of glucose is completely inhibited, and on the other the diffusion or secretion of creatinine and possibly also of sucrose is greatly reduced. That we have been unable to abolish completely this apparent diffusion of creatinine, and in this respect differ from the Bellevue Hospital workers, does not seriously affect the conclusions which one feels compelled to draw. Nor does our finding that in the absence of phlorrhizin the sucrose clearance exceeds that for xylose.

It is not difficult to believe that, in the phlorrhizinized kidney, some permeability of the tubules for creatinine is still present.

But whether or not this explanation of the facts be accepted, and it is difficult to put forward any other, it would seem quite certain that the figure representing the xylose clearance has a real meaning in the kidney. The same figure can be arrived at by a study of the excretion of either



sucrose or glucose under the influence of phlorrhizin. A rough approximation to it can be obtained from the creatinine clearance under phlorrhizin or the sucrose clearance without phlorrhizin.

Further support for the importance of this figure is contained in the succeeding paper, in which it is shown that precisely the same value can be arrived at by the use of an entirely independent substance, having widely different chemical properties [Cope, 1933]. So far as we are aware no other value is known for the kidney which can be arrived at in even two ways, without irreversible damage to that organ.

We feel, therefore, that the phenomena presented by the excretion of these substances well merit further investigation, and that they offer great hope for the development of a renal function test along rational lines.

#### SUMMARY.

The excretion of the sugars xylose and sucrose has been compared with that of glucose after phlorrhizin, and with creatinine with and without phlorrhizin in the rabbit's kidney.

In confirmation of previous workers the clearances for all three sugars have been found equal after phlorrhizin.

In the absence of phlorrhizin the sucrose clearance was about 30 p.c. higher than the xylose clearance.

The creatinine clearance may be from 0 to 50 p.c. higher than the sucrose clearance, but is never lower. This difference is not appreciably affected by phlorrhizin.

Creatinine clearance averages 55 p.c. above xylose clearance, but falls to only 20 p.c. above it after phlorrhizin. It has never been found equal to the xylose clearance.

Creatinine clearance is roughly proportional to the sucrose clearance over a wide range of clearance values. The ratio between the two is not appreciably influenced by the concentrations of either in plasma or urine, nor by the action of urea.

If allowance be made for varying renal activity, an approximately linear relation can be found between plasma concentration and rate of excretion for both creatinine and sucrose.

The interpretation of these findings is considered.

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## THE EXCRETION OF CYANOL BY THE MAMMALIAN KIDNEY.

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THE interesting experiments forming the basis of claims recently put forward for the sugars xylose and sucrose as indicators of glomerular filtration rate in the kidney have been in part confirmed and discussed in the previous paper [Cope, 1933].

It became of interest also to compare the excretion of these sugars with that of another substance of quite different type which had also been claimed to behave similarly in the kidney—the dye “Cyanol extra.”

The experimental phenomena presented by xylose and sucrose have already been stated elsewhere and need not be further repeated here, but it is convenient briefly to summarize the evidence relating to cyanol.

Cyanol belongs, according to Höber, to the group of highly diffusible, lipoid insoluble acid dyestuffs. It is readily excreted in the urine of most animals after subcutaneous or intravenous injection. But when injected into the blood stream of the fish *Lophius piscatorius*, whose kidney has no functional glomeruli, it appears only in traces in the urine, although phenol red injected at the same time is readily excreted [Höber, 1930].

Similarly, when the glomeruli of another fish, the sculpin, are rendered inactive by several doses of phlorrhizin, injected cyanol does not appear in the urine in appreciable amount [Marshall and Grafflin, 1932].

Again, when supplied to the tubules of the frog's kidney by perfusion through the portal vein, it is excreted in only small amount at low concentration, although when perfused by the aorta through the glomeruli, it is much more rapidly excreted and more highly concentrated in the urine [Schulten, 1925]. In view of these and other similar results Höber considered it was proved that cyanol is a purely glomerular excretory product, not being appreciably influenced by the tubule cells.

Descombes [1932] accordingly attempted to show that both cyanol and creatinine behave in identical manner in the mammalian kidney, his purpose being to obtain further support for Rehberg's contention [1926] that creatinine also is excreted only by the glomeruli. The conclusions which he draws are, however, scarcely justified by the results which he presents. No blood analyses were made, and it was presumably assumed without experimental proof, that after intraperitoneal injection both were absorbed at equal rates into the blood stream. Further, as Descombes himself freely admits, the technical difficulties of estimating both creatinine and cyanol simultaneously in the same solutions are very great owing to the interference of the blue colour with the orange of the Jaffe reaction for creatinine. He found, however, that there was a fairly good parallelism between the rates of excretion of cyanol and creatinine, but his conclusion that this indicates a similarity of excretory mechanism for both we cannot accept on his evidence.

Frey also [1932] has, in a few experiments, used cyanol as a measure of glomerular filtration rate, but adequate comparisons with other methods were not made. He obtained, in one experiment on a rabbit under urethane, figures which indicate a cyanol clearance in two periods of 168 and 300 c.c. per hour. Such figures are rather below those usually obtained by the use of sucrose or xylose, but this may well be due to the effect of the urethane.

In the work here to be presented cyanol excretion has been compared in rabbits with that of xylose, sucrose and glucose.

#### METHODS.

The methods used in the sugar analyses have been discussed elsewhere [Cope, 1933] and were used here also without modification. Somogyi copper filtrates of both blood plasma and diluted urine were used in all cases.

The method used to determine the concentration ratio for cyanol calls for explanation however. For a supply of this dyestuff we are indebted to the generosity of Messrs Bayer Products, who assure us that it is similar to that used by Prof. Höber himself.

The dye dissolves freely in water to give a deep blue solution. Spectroscopically examined a weak solution shows a well-marked absorption band in the region 590–620  $\mu\mu$ . It differs markedly from hæmoglobin in showing no significant absorption in the blue. This fact has been made use of in estimating cyanol in plasma. It was not found

possible to obtain plasma samples from rabbits, which were so free from hæmolysis or slight yellow tinge that they could be accurately compared in a colorimeter against an aqueous cyanol standard. The yellow tinge of the plasma has accordingly had to be compensated by the insertion of a cyanol-free plasma on the other side of the colorimeter. In doing this, the Leitz universal compensating colorimeter has been found to be of the greatest value.

It has been employed as follows: After obtaining a satisfactory centred light source, the cyanol-containing plasma sample is placed, without further treatment than good centrifuging, and without dilution, into a micro cup on the left side of the middle stage of the colorimeter. A cyanol-free plasma, obtained preferably from the same rabbit before it has received the cyanol injection, is placed in the corresponding right cup. In the top stage, where micro cups are not necessary, water is placed in the left-hand cup and a suitable cyanol standard on the right side. The coupled plungers of this upper pair of cups are then lowered to the bottom so that the light passes through the two plasmas only. The bottom stage of the colorimeter is left empty. The blue light filter (No. 2) which transmits mainly at  $450\text{ }\mu\mu$  is then inserted in the system. Now, with the left-hand blue plasma adjusted to a definite depth, usually 5 or 10 mm., differences in the intensity of the two halves of the visual field are due partly to opalescence but mainly to the yellow colour of the plasma itself. The right-hand cyanol-free plasma is now adjusted until the light intensity is equal on the two sides of the field. Next, without moving either plasma, the light filter is changed to a red (No. 9), transmitting at a wavelength of about  $600\text{ }\mu\mu$ . The field again become unequal owing to the light absorption of the cyanol in this part of the spectrum, and then, still using the same red filter, the upper coupled plungers are raised until equality of the fields is restored, and the comparison between plasma and aqueous cyanol standard is thus made.

This procedure, which is much simpler in practice than in description, has appeared to give quite satisfactory results even when hæmolysis was sufficient to give a perceptible greenish tinge to the cyanol-containing plasma.

Actually, in these experiments, determination of the cyanol concentration ratio was of greater importance than a knowledge of the absolute cyanol concentrations. Hence, in order to minimize experimental errors, the corresponding urine suitably diluted was used in the upper cups, in place of a standard of known concentration. In this way the cyanol concentration ratio has been measured directly. The absolute concentration

in the urine was found by comparing a diluted urine sample with an aqueous standard containing 1 mg. cyanol per 100 c.c. Plasma cyanol concentrations were then calculated from the urine concentration and the concentration ratio.

### RESULTS AND DISCUSSION.

Preliminary comparisons of the cyanol clearance with that of sucrose revealed that although they are of similar magnitude, the identity is not complete. The sucrose clearance tends to be some 25 p.c. higher than the cyanol clearance.

Urine vol. c.c. per hr.	Sucrose			Cyanol		
	Plasma	Urine	Clearance	Conc. ratio	Clearance	Cyanol Sucrose
26.8	137.4	2781	543	15.3	410	0.75
21.1	136.7	3520	545	25.1	530	0.97
7.2	149.9	12507	601	73.0	525	0.875
6.9	140.3	12203	599	69.0	475	0.795
					Mean	0.85

But since it had been previously found that xylose has a clearance some 20 p.c. lower than sucrose in the absence of phlorrhizin, a direct comparison of xylose with cyanol was evidently likely to give a closer correspondence between the two clearances. This has accordingly been made, and although occasional deviations have occurred, the tendency for the two clearances to be identical has been definite. In a series of thirteen such comparisons the value of the ratio cyanol clearance/xylose clearance has been within the limits of 0.975 and 1.03 in ten, and in the remaining three was 0.85, 0.83 and 0.79.

TABLE I. Comparison of xylose and cyanol clearances.

Urine vol. c.c. per hr.	Xylose		Cyanol		Clearances		Ratio Cyanol Xylose
	Plasma	Urine	Plasma	Urine	Xylose	Cyanol	
45.6	133.0	1170		8.6*	401	392	0.98
21.2	116.0	2245		20.0*	411	424	1.03
18.0	140.0	2180	2.4	36.3	280	272	0.975
11.3	115.5	3950	2.0	66.6	386	377	0.975
9.33	139.0	5150	3.16	117.0	345	345	1.00
10.2	131.5	5025	2.67	100.0	390	382	0.98
8.13	100.4	3723	2.96	109.0	301	299	0.995
1.96	89.7	7019	2.92	195.0	153	131	0.856
16.8	85.3	1819	2.76	48.8	358	297	0.83
10.6	68.1	2461	1.81	64.5	384	378	0.98
						Mean	0.96

\* Concentration ratio.

TABLE II. The effect of phlorrhizin on the cyanol clearance.

Exp.	Urine vol. c.c. per hr.	Xylose		Glucose		Cyanol		Clearances			Glucose Xylose	Cyanol Xylose
		Plasma	Urine	Plasma	Urine	Plasma	Urine	Xylose	Glucose	Cyanol		
1	19.1	155.0	3220	104.1	2327	9.48	76.4	397	426	154	1.07	0.39
	15.7	143.0	2910	111.3	2463	9.51	88.1	320	347	145	1.08	0.455
	10.0	153.5	6420	149.4	Trace	3.32	109.0	418	—	328	—	0.79
2	18.3	115.0	3678	147.2	3495	2.99	41.4	583	433	252	0.74	0.43
	15.8	77.3	2563	145.4	4816	2.68	32.5	525	523	191	1.00	0.36
	7.95	128.3	5266	—	Trace	2.67	110.0	326	—	327	—	1.00
3	13.65	120.2	3028	146.1	2903	3.86	58.0	344	272	205	0.79	0.60
	12.8	98.2	2451	151.9	3750	4.05	44.0	320	316	139	0.99	0.43
	12.3	174.0	5400	—	Trace	3.67	111.0	382	—	373	—	0.98
4	17.5	135.0	3230	150.6	2818	3.39	47.7	419	327	246	0.78	0.59
	18.0	103.8	2175	166.2	3810	3.31	32.8	377	380	178	1.01	0.47

This marked tendency of the value of the ratio to be unity would seem too great to be attributed to chance, and it appears justifiable therefore to attribute the three discrepant values to the existence of some interfering process which is not usually present.

On attempting to extend this comparison to include glucose in the phlorrhizinized kidney, entirely unexpected results were obtained. For while the glucose clearance rose to equal the xylose figure, that for cyanol fell very considerably to a value much below the other two. Its behaviour therefore was practically the opposite of that of glucose. Taking only those values obtained during full phlorrhizinization, as indicated by the value of the glucose clearance/xylose clearance ratio, the mean value of the ratio cyanol clearance/xylose clearance is then only 0.42 as compared with a mean of 0.95 for all the thirteen values of the same ratio obtained in the absence of phlorrhizin.

The inhibitory effect of phlorrhizin on cyanol excretion is thus very large, and is in fact readily apparent by simple inspection of the excreted urines.

In Fig. 1 it is compared with the behaviour of the sugars and creatinine towards phlorrhizin.

The interpretation of these findings is not easy, nor is it rendered any easier by the most recent work of Höber and Meirowsky [1932] on the excretion of cyanol in the frog's kidney.

The correspondence of the cyanol and xylose clearances in the normal kidney would have been in good accord with the view that both are excreted by the same mechanism, *i.e.* by glomerular filtration alone, were it not for the doubt cast on his own original interpretation by Höber himself in the above-mentioned paper. For he has recently found that, although cyanol behaves as though excreted only by the glomeruli when the kidney is perfused with a modified Ringer solution, yet when perfused with blood or a hæmoglobin containing solution, the tubule cells appear to be permeable to cyanol.

If this be true also of the mammalian kidney, then the close correspondence of the cyanol and xylose clearances will have to be regarded as a coincidence, although a rather remarkable one. It would then be possible to explain the inhibitory effect of phlorrhizin as due to a cessation of excretion of that fraction of the cyanol excreted by the tubules. But on such a view the remaining glomerular fraction which survives the inhibiting effect of phlorrhizin would be only 40 p.c. of the amount to be expected from the various sugar clearances. This in turn might be attributable to an adsorption of some of the dye in the plasma, rendering it



indiffusible in the same manner as is known to occur with phenol red. With this possibility in mind an attempt was made to determine what fraction of the plasma cyanol was freely diffusible. No appreciable amount of cyanol could, however, be dialyzed out through collodion membranes from plasma taken from rabbits previously injected with the dye either with or without phlorrhizin. This point must therefore be left undecided.

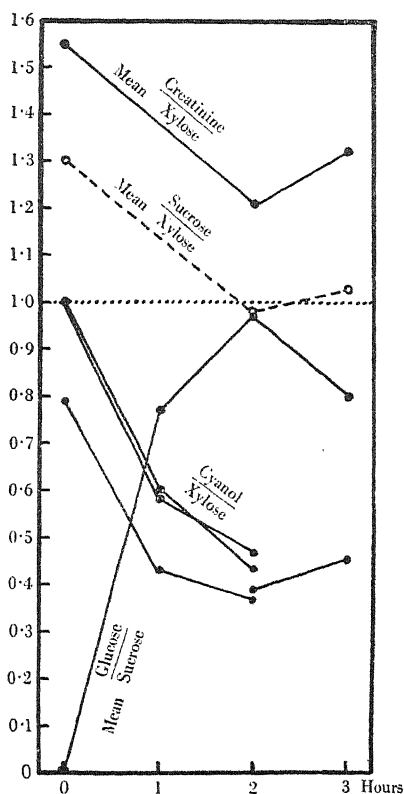


Fig. 1. Effect of phlorrhizin on the cyanol clearance compared with the effect on sugars and creatinine.

But even if Höber's original view that cyanol is a purely glomerular product be correct, then, although the similarity of the cyanol and xylose clearances becomes explicable, the anomalous behaviour of cyanol in the phlorrhizinized kidney still requires some other explanation.

It is clear therefore that further work on the excretion of cyanol is required before these phenomena can be adequately explained.

## SUMMARY.

The excretion of the dye "Cyanol extra" has been compared with that of sucrose, xylose and glucose in the rabbit's kidney.

The sucrose clearance has been found to be some 25 p.c. higher than that for cyanol. The xylose clearance was equal to the cyanol clearance within the limits of experimental error in ten out of thirteen comparisons.

Under the action of phlorrhizin the glucose clearance rises to equal the xylose clearance, but the cyanol clearance falls considerably and then averages only 42 p.c. of the xylose or glucose clearance.

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INFLUENCES WHICH AFFECT THE FORM OF THE  
RESPIRATORY CYCLE, IN PARTICULAR THAT  
OF THE EXPIRATORY PHASE.

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IN a communication to this *Journal* [Hammouda and Wilson, 1932] the respiratory cycle was described as composed of three phases, the third being the expiratory pause shown as a horizontal line on the graph. We drew attention to the form of the cycle in decerebrate or chloralosed animals, since we believed that it represents correctly the cycle of activity of the central nervous mechanism and that the absence of change in volume of the lungs during the pause proves that the initiation of the inspiratory act could not be due, as had been commonly supposed, to an excitatory stimulus arising in the lungs. The results seemed to show that the respiratory centre governs only the rate and depth of inspiration, expiration being an act of passive recoil.

In a paper published subsequently Sharpey-Schafer [1932] states that there is no pause in the expiratory phase of the normal human cycle, and he holds that an active factor forms an essential part of this phase.

Although the type of cycle as described by Sharpey-Schafer is that most commonly seen, it is nevertheless an undoubted fact that a pause as definite as that seen in animals (Gad [1879] in the conscious rabbit, Head [1889] in the same animal under chloral and ourselves in decerebrate or chloralosed dogs) does occur in the human subject under normal conditions of life. We discussed this question briefly in the appendix to Section I of the paper referred to above. Reference was made to the published records of other authors in which human respiratory cycles are illustrated which show a definite and well-marked expiratory pause. The papers referred to were those of A. Mosso [1878] showing the cycle of a human subject when asleep, Luciani [1911] of a man at rest but awake, and of Haldane and Priestley [1905] in one of their subjects whose breathing was recorded with the body plethysmograph. Later in this paper a figure is shown (Fig. 7) demonstrating this fact. It is evident that

the occurrence of a pause during which the volume of the lungs remains unchanged does not imply anything abnormal in the action of the respiratory centre.

We hope to be able to show that the presence or absence of a pause in the expiratory phase is not related to any active factor apart from the probable reflex control of the frictional resistance of the air passages to the exit of air. An active factor in expiration should be taken to mean a muscular action present in normal breathing and necessary to the discharge of air from the lungs. That such action is not essential is shown by the Drinker respirator. With this apparatus breathing can be maintained at a normal rate in persons with complete paralysis of the respiration, inspiration being effected by the production of a negative pressure external to the body, expiration by passive recoil.

In a paper read recently at a meeting of the Physiological Society, one of us (W. H. W. 1933) has drawn attention to the fact that records of the intra-abdominal pressure do not show any evidence that the abdominal muscles act otherwise in respiration (except in forced expiration) than as a passive elastic membrane, the tension of which is increased by the descent of the diaphragm and which recoils when contraction of the diaphragm ceases. There is, in fact, no indication of any relaxation of tone of the abdominal muscles during normal inspiration such as might be expected to occur if these muscles behaved as the physiological antagonists of the diaphragm. The older view of the part taken by muscular action in expiration was that this was analogous to inspiration and was controlled by an expiratory centre in the medulla. It is now thought that such actions are proprioceptive reflexes originating in the inspiratory muscles. This clearly supports the view that the respiratory centre is essentially inspiratory in function.

A. Fleisch [1928] believes, from his observations on human subjects of the reactions to sudden changes of intra-pulmonary pressure in either direction or to closure of the air inlet during expiration or inspiration respectively, that such proprioceptive reflexes are of importance in normal breathing<sup>1</sup>. Such reactions should continue with diminishing

<sup>1</sup> It may be pointed out that A. Fleisch and other observers have drawn deductions from the results of observations on human subjects using procedures such as the sudden closure of the air outlet or the establishment of pressures resisting expiration which exceed that normally existing in the air passages or can be overcome by the effect of elastic recoil (with normal expansion of the chest about 5 mm. Hg or 6.5 cm. H<sub>2</sub>O). The conditions so established can, in fact, only be overcome by muscular action which would not ordinarily be necessary. The reactions to such procedures might very well be regarded as demonstrating the mechanism whereby the breathing is adapted to conditions which may arise but which are abnormal: it is, however, doubtful whether they should be regarded as demonstrating the occurrence of reflex actions taking part in normal breathing.

effect throughout the expiratory phase. No evidence of this can be seen in the cycle of tracheal breathing in decerebrate animals nor, if slight inflation of the lungs be effected during the inspiratory phase, is the termination of inspiration followed by any reversal which is not explained by the release of the passive recoil forces.

Adrian [1933] has found evidence of electrical activity during expiration in the internal intercostals and triangularis sterni of chloralosed cats or rabbits. The activity is of the same kind as that of the externals in inspiration. Adrian informs us that as a rule, however, only the intercostals of the front of the thorax contract regularly, farther back they maintain an even state of contraction or are relaxed. With anæsthetics other than chloralose the expiratory muscles are usually inactive, while in decerebrate animals, in which the effect of an anæsthetic is eliminated, these muscles are sometimes but not always active.

It is evident from Adrian's observations that, under certain conditions, the internal intercostals are active, and it might be expected that when this is so the effect would be shown in the form of the cycle.

Our failure to observe evidence of this on the volume curve may be due to slight differences in the resistance of the air passages due, for example, to differences in the diameter of the tracheal cannulæ employed in our own and in Adrian's experiments.

The fact that the activity observed by Adrian only occurs regularly under certain conditions, and even then does not appear to affect the intercostal muscles throughout suggests that in normal breathing these muscles may, by the maintenance of a constant tone, act as do the abdominal muscles merely as a part of the elastic recoil system.

The main function of the abdominal muscles is to support the weight of the viscera. By their elastic recoil they assist expiration. The intercostal muscles have a similar function in supporting, by their tonic contraction, the intercostal spaces against the negative intra-thoracic pressure. It is possible therefore to regard the internal intercostals as having in normal breathing the same passive rôle as that of the abdominal muscles.

This view seems to be supported by Sharpey-Schafer and Macdonald's observations [1925] on a costo-sternal hoop formed of three ribs, with the nerves and blood vessels intact but with complete separation of their muscular attachments above and below. The thorax is open. When artificial respiration is stopped, rhythmic up-and-down movements (inspiratory and expiratory) of the ribs begin.

They note that while the active contraction of the external intercostal muscles can be observed readily during inspiration no such active con-

traction of the internal intercostals can be observed during expiration, except during the exaggerated movements of dyspnoea. They found, in fact, no evidence that the return of the ribs to their original position in normal breathing was not due to their elastic recoil from the condition of tension produced by the raising of the ribs in inspiration. Thus there does not seem to be any evidence that these muscles undergo relaxation of tonus reciprocal to contraction of the external intercostals, or that they show an increase in their tonic contraction which is effective in assisting expiration.

The apparent difference between the cycle described by Hammouda and Wilson [1932] in chloralosed and decerebrate animals and that usually seen in man, makes it necessary to consider to what this difference is due, and whether it implies a nervous reflex control different from, or additional to, that which they were able to demonstrate under the conditions of their experiments.

In their observations the following factors were absent: (a) the frictional resistance of the upper air passages (normally increased at the larynx and nostrils in expiration) to the inflow or outflow of air; (b) the possible effect of reflex stimuli arising in the mucosa of that region; (c) the effect of influences arising in the nervous system above the mid-brain (reflex or cortical) which may be necessary in the adaptation of the breathing to changes of posture or active bodily movements.

The observations recorded below have been designed to elucidate these questions, by determining (a) the form of the cycle in the same animal breathing through the normal channels (glottis, etc.) as compared with breathing through a tracheal cannula; (b) the effect of a resistance to the outlet or inlet of air; (c) the effect of an anæsthetic, and (d) the effect on the cycle or rhythm of breathing of any reflexes which might arise in the upper air passages.

## I. COMPARISON OF THE CYCLE IN TRACHEAL AND MASK BREATHING.

*Method.* Fig. 1 shows the apparatus devised for this purpose. Chloralosed or decerebrate dogs were used. The trachea was opened sufficiently and the two-way tracheal end of the cannula (*B*, Fig. 1) tied in. It will be seen that air can therefore pass directly to the upper air passages or, if the mask exit is blocked and the tracheal open, directly to the tracheal. The rubber face-piece was modelled to fit tightly to the muzzle of a dog of the size usually employed. The face-plate *b* and

attached face-piece is first fixed in position by two tapes, not shown in the diagram, attached to the sides of the mask and tied behind the head of the animal. In actual use the nose of the dog is much closer to the opening in the plate *b* than is shown, the dead space being then from 20 to 30 c.c. The opening in the plates *a* and *b* is large enough to prevent

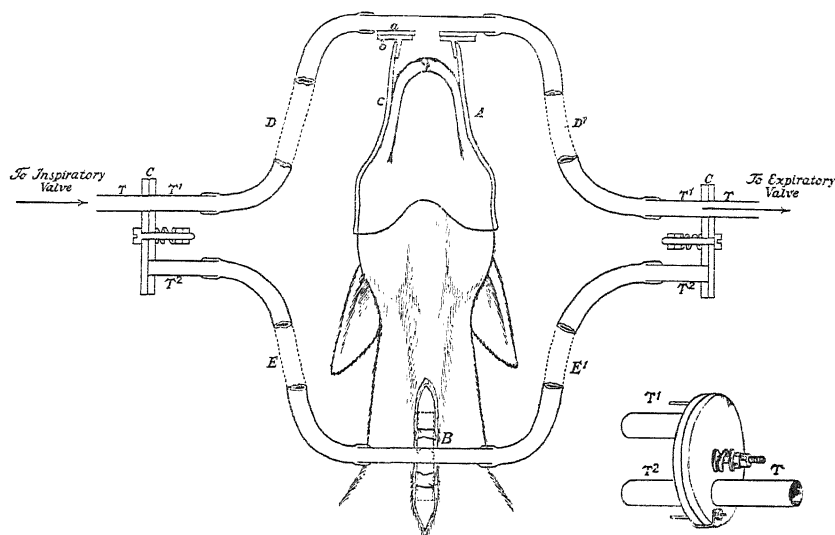


Fig. 1. Apparatus for alternative tracheal and mask breathing. *A*, mask; *a*, detachable brass plate, carrying inlet and exit tubes, which can be clipped on to *b* to a flange on which the conical rubber face-piece *c* is attached; *D*, *D'*, inspiratory and expiratory rubber connections from mask to air switches; *B*, two-way cannula with *T'* connection to *E*, *E'*, inspiratory and expiratory rubber connection from trachea to air switches; *C*, *C'*, air switches *in situ*. Inset, same shown in perspective. *T*, *T'*, connection to inspiratory and expiratory valves; *T''*, connection to mask; *T'*, connection to trachea. In the diagram the switches are shown in position for mask breathing; in the drawing inset they are shown in position for tracheal. A catch is seen on the outer plate of the switch and two stops on the inner plate. The outer plate can be rotated and is arrested by one stop opposite *T''* by the other opposite *T'*. The tubes and connections are all of  $\frac{5}{8}$  in. diameter.

pressure on the nostrils or their surroundings. The plate *b* also carries a short metal tongue which is passed between the incisor teeth partly to steady the mask, partly to keep the jaws slightly open. Between the plates *a* and *b* is a rubber washer which makes the joint airtight.

The tubes *E* and *E'* from the tracheal cannula, and the plate *a* of the mask, are attached when the preparations for the experiment are com-

plete. For observations where no great changes of pressure occurred the simple rubber face-piece gave quite satisfactory volume records.

In the conduct of an experiment the air tubes  $T^1$ ,  $T^2$  of the air switches are held firmly in clamps placed on either side of the animal. To switch over from the mask or *vice versa* (Fig. 2), the observer, holding the tube  $T$  of each switch, one in the right the other in the left hand, rotates them rapidly in the correct direction at any desired moment in the cycle (by observation of the kymograph record or signal from a colleague). It will be seen from the figures that this can be done without any appreciable delay. Observations with this method have been made on the effects of inflation and deflation of the lungs with tracheal and mask breathing, the tubes from the trachea and mask respectively being passed through the cover of the chamber described by Wilson and Hammouda [1928]. The results were identical whether the breathing was by the normal channels (mask) or by the tracheal outlet: indicating that the passage of air in either direction over the mucosa has no effect.

Using the method described above the effect on the form of the cycle of switching over from tracheal breathing to breathing through the normal channels has been examined. Examples of the results are shown in the following figure (Fig. 2).

From Fig. 2 it will be seen that the general form of the cycle is the same whether the air entry and exit is through the normal passages or by a tracheal opening. The measurements given below the figure show, however, certain quite distinct differences in the phases. The duration of the whole cycle (rate of breathing) is little affected. In tracheal as compared to mask breathing the depth of inspiration is greater, its duration in some cases slightly less. The first phase of expiration (*b*, *c*) is slightly shortened, the expiratory pause is of longer duration. The line of expiration (phase 1) is less oblique and less curved.

It will be shown later that these differences are explained by a slightly greater frictional resistance to the air passing through the upper air passages in both inspiration and expiration. The resistance offered by the upper air passages is evidently very small in animals under the conditions described, and in these observations appears to be greater in the inspiratory than the expiratory phase. It is evident from Fig. 2 B that the cycle after vagotomy is the same whether the breathing takes the ordinary channels or is through the tracheal outlet; the only difference between the two curves (*M* and *T*) being a slightly greater obliquity and rounding of the first phase of expiration in the mask breathing as compared to the tracheal.



In Fig. 2 C a similar observation is shown made on a dog under chloralose, the breathing being switched over from tracheal to mask. The whole cycle was slightly shorter and the depth of inspiration was greater in proportion to the duration than in Fig. 2 A.

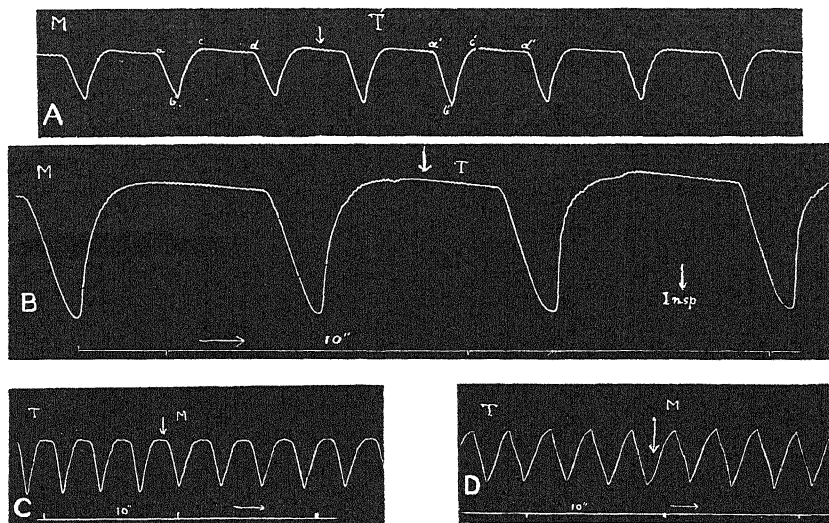


Fig. 2. Comparison of cycle in tracheal and mask breathing. **A.** Dog. 5 kg. Morphine, 0.1 c.c. of 5 p.c. sol. per kg. C. and E. Decerebration immediately in front of ant. colliculi 3 hours before time of observation. A before, B after, section of cervical vagi. *M*, breathing through normal passages (mask); *T*, through tracheal cannula; arrow shows moment of change-over from *M* to *T*. Means of cycles before and after switching over. Rate of breathing: 19.5 per min. in both *M* and *T*. Duration of inspiration: *M*, 0.75; *T*, 0.67 sec. Duration of expiration: 1st phase, *M*, 0.9, *T*, 0.8 sec.; pause, 1.46; 1.64 sec. *a, b*, inspiration; *b, c*, expiration, 1st phase; *c, d*, pause; *a', b', c', d'*, same for tracheal breathing. **B.** After section of vagi in neck. Rate of breathing: 7.7 per min. **C.** Dog. 5 kg. Morphine and C. and E. as above. Chloralose 0.07 g. per kg. *T*, tracheal breathing; *M*, mask breathing; arrow shows moment of change-over. Rate of breathing: *T*, 21; *M*, 19½ per min. Duration of expiration: *T*, 2.05; *M*, 2.3 sec. Duration of inspiration the same in *T* and *M*. **D.** Dog. Chloralose. Abnormal type of cycle resembling that sometimes described as normal for man. Rate of breathing: *T*, 21; *M*, 20 per min. Duration of expiration: *T*, 1.74; *M*, 1.87 sec. In all records read left to right; inspiration down, expiration up.

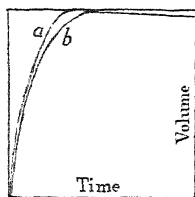
In this case, although the duration of inspiration was little affected by the change, the depth was definitely diminished in mask breathing as compared to tracheal. The expiratory phase shows a similar but more marked change than in Fig. 2 A. The pause being almost abolished.

In Fig. 3 the expiratory phase of typical examples of cycles of mask and tracheal breathing respectively in the two cases have been brought to the same duration and volume scale.

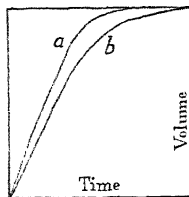
In the curves in Fig. 3, *a* is that of the expiratory cycle in tracheal breathing, *b* that in breathing through the normal channels.

The curves may be compared with those given later, in which an artificial resistance has been introduced in the exit of air with tracheal breathing.

The observation recorded in Fig. 2 D is from a chloralosed dog. The cycle closely resembles in type that described by Schafer [1932] as being a common normal type in man (*vide* that author's Fig. 2). We have



(1) Graph A, Fig. 2.



(2) Graph C, Fig. 2.

Fig. 3. Form of the expiratory phase in tracheal and mask breathing. (1) Expiratory phase of cycles in Fig. 2 A. *a*, tracheal breathing; *b*, mask breathing. (2) The same for two cycles of Fig. 2 C. (1) is from a decerebrate, (2) from a chloralosed animal. The depth and duration have been brought to the same level in each case.

occasionally, though rarely, met with this form in animals under chloralose, but in our experience it is never seen after decerebration. It will be observed that there is no change in the form of the cycle on switching over from tracheal to mask breathing. There is no significant alteration either in the depth of inspiration or the duration of either phase. It is evident that the absence of the pause, and the regular and continuous rise in the line of expiration, are in no way connected with the effects of frictional resistance or reflex influences arising in the upper air passages, and it seems unlikely that the resistance of the bronchi could explain the form. The fact that the line of expiration closely resembles that of inspiration suggests that an active element exists in expiration in this type of cycle which is absent in that commonly seen. This type of cycle is the same as that described by Gad as occurring in the conscious rabbit breathing through a mask, before the animal has become quiescent after the first emotional disturbance. It also resembles the cycle of panting (except in rate) which one of us [Hammouda, 1933] has shown to be

related to the action of a centre situated in the thalamic region. In the emotional type of cycle described by Gad and in panting both expiration and inspiration are active processes.

That the subject whose respiratory cycle is recorded in Schafer's figure referred to above was conscious of his breathing and that the rhythm therefore cannot be regarded as normal is suggested by the varying depth of inspiration and particularly by the irregularity in the point reached in expiration, the base line of expiration being normally a straight line unless affected by postural changes.

## II. EFFECT OF RESISTANCE TO THE ENTRY OR EXIT OF AIR ON THE FORM OF THE EXPIRATORY AND INSPIRATORY PHASES RESPECTIVELY.

From the observations recorded in Fig. 2 A, C, it is evident that certain changes take place in the duration and form of the phases which may probably be explained by a greater frictional resistance to the passage of air through the upper air passages as compared to the tracheal outlet. The following observations are designed to show the effect of resistances of known amount introduced into the expiratory or inspiratory air way to a tracheal cannula.

*Method.* A cannula is tied into the trachea and to this is attached a T tube, one branch of which goes to the expiratory the other to an inspiratory valve. Between the cannula and the valves a connection is made with a mercurial manometer which thus registers the resistance against which the animal is inspiring or expiring. The valves are connected in the usual way with a 10-litre bottle and a bellows volume recorder. The rubber tube connecting either the inlet or exit valve with the air reservoir (whichever phase it is desired to study) is passed under a clamp, by depressing the handle of which the tube is compressed. An adjustable stop is provided by which the descent of the clamp can be arrested at any point. The passage of air can thus be obstructed to any desired extent. The exit or inlet tube having been placed in position, a few normal cycles are recorded, and the clamp is then closed at any suitable point in the cycle.

### 1. *Effects of resistance to inspiration.*

In Fig. 4 the effects of four different degrees of resistance are shown before and after section of the vagi. The curves in the different cases have been analysed and are represented in a semi-diagrammatic form in the two graphs (III, IV) below the records.

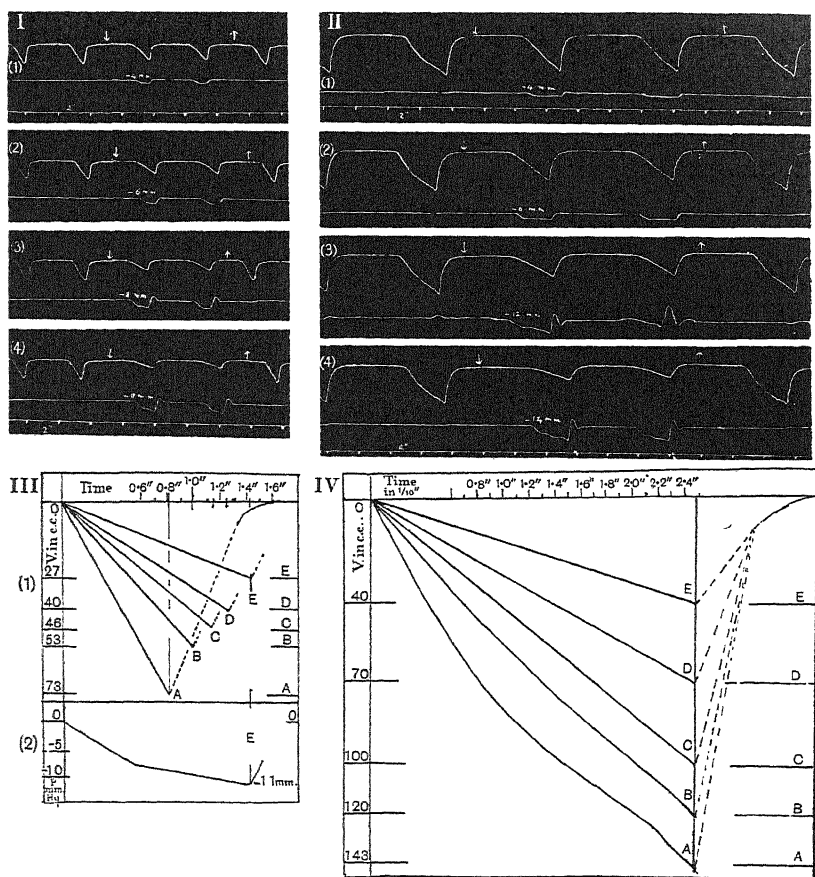


Fig. 4. Effect of resistance to inspiration on the cycle. Dog. 5 kg. Morphine, C. and E. and chloralose. I. Vagi intact. Resistance: Graph (1) - 4 mm.; (2) - 6 mm.; (3) - 8 mm.; (4) - 11. II. Vagi cut. Resistance: Graph (1) - 4 mm.; (2) - 6 mm.; (3) - 12 mm.; (4) - 15 mm. In all graphs read left to right; inspiration down, expiration up. Downward arrow shows point at which tube to inspiratory valve was clamped. Upward arrow shows release of clamp. The cycles affected are shown on the manometer record by fall of pressure. Upper tracing, volume record; lower, Hg manometer. III. Semi-diagrammatic representation of the inspiratory phase in the four cases before vagotomy. IV. The same after vagotomy. In III, graph (2) represents the pressure change during inspiration in graph I (4).

Vagi intact (I, III)					
	A.	B.	C.	D.	E.
P	0	- 4	- 6	- 8	- 11
T	0.8	1	1.15	1.25	1.45
V	73	53	46	40	27
Vagi cut (II, IV)					
	A.	B.	C.	D.	E.
P	0	- 4	- 6	- 12	- 15
T	2.45	2.45	2.45	2.45	2.45
V	143	120	100	70	40

P = resistance to inspiration in mm. Hg; T = duration of inspiration in sec.; V = depth of inspiration in c.c.

The manometer record of E only is shown in graph III, the pressure changes in the other cases being similar but of lesser extent.

An inspection of the results illustrated in Fig. 4 shows the following effects of resistance to inspiration in animals under the conditions of the experiments: (*a*) the rate of breathing (total duration of cycle) is unaffected; (*b*) the depth of inspiration is diminished in proportion to the resistance; (*c*) the duration of inspiration is increased; (*d*) the pressure curve (with constant resistance) shows at first a rapid, followed by a slower but continuous increase in negative pressure indicating a gradual increase in the force of the inspiratory muscular contraction which, in view of the progressively increasing resistance to expansion in inspiration under all circumstances, evidently represents the normal course of action of these muscles.

After section of the vagi (IV) the results are the same with one exception, namely that the duration of the inspiratory phase is identical in all four cases, which would indeed be expected if it be remembered that the duration of this phase after vagotomy represents the duration of the discharge from the respiratory centre unaffected by reflex influences reaching it from the lungs.

A further point of interest appears from a comparison of the relative effect of resistance to the inspiratory phase before and after vagotomy. Hammouda and Wilson [1932] drew attention to the fact that the depth reached in a given time in the inspiratory phase after vagotomy is the same as in the normal cycle before vagotomy, although in the former owing to the absence of the inhibitory reflex excited by expansion the discharge from the centre (and inspiration) continues for the full period. This observation is confirmed by the results shown in Fig. 4. If curves A, B and C be compared in graphs III and IV of that figure, A being the inspiratory phase without resistance, B in the two cases with — 4 and C with — 6 mm. respectively, the depth reached in the same lapse of time, namely 0.8, 1.0 and 1.15 sec., is almost exactly equal.

These results support the view we expressed that, while the whole central mechanism is under the influence of the chemical stimulus, the rate of the rhythm and the duration of the discharge are controlled by stimuli arising in the lungs, but the force of the discharge and so the strength of the contraction of the inspiratory muscles is not under this reflex control but is dependent on the state of chemical excitability of the centre.

## 2. The effect of resistance to expiration.

In the observations illustrated in Fig. 5 the effect of three different degrees of obstruction to the outlet is shown (I (1), (2), (3)). In

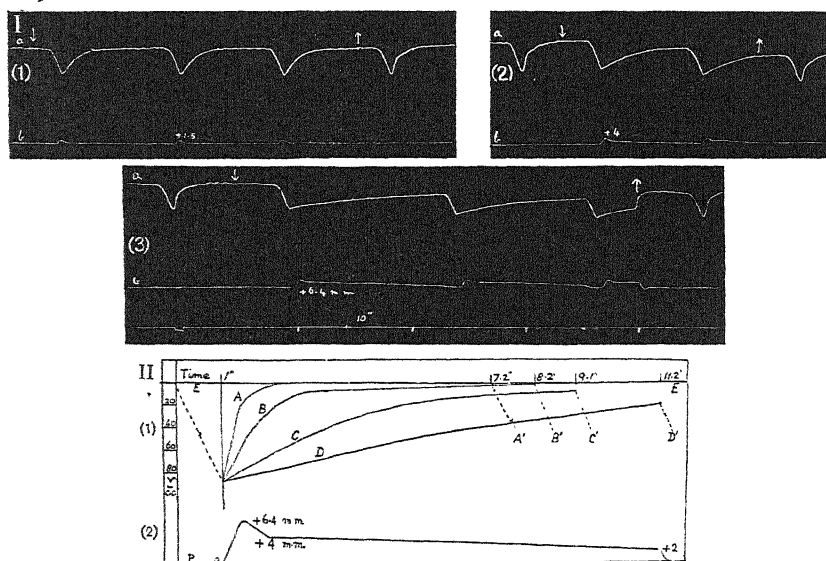


Fig. 5. Effect on the cycle of resistance to expiration. Dog. 4 kg. (same animal as in Fig. 6) after chloralose, 0.07 g. per kg. I. Record of results. Initial resistance: Graph (1), 2.5 mm. Hg; (2) 4 mm. Hg; (3) 6.4 mm. Hg. *a*, volume record; *b*, Hg manometer record. Downward arrow shows point at which rubber connection to expiratory valve was compressed. Upward arrow shows release of clamp. II. Semi-diagrammatic representation of cycles in the three cases. Broken line -----, inspiration; full line ———, expiration. *E*, *E*, base line of expiration; *P*, base line of manometric pressure. *A*, normal expiratory phase without resistance (graph 1); *B*, expiratory phase with resistance = 2.5 mm. (graph 2); *C*, expiratory phase with resistance = 4 mm.; *D*, expiratory phase with resistance = 6.4 mm. *A'*, *B'*, *C'*, *D'* = termination of expiratory phase. The curves have been plotted from tracings and measurements. The duration of the normal cycles in the three cases was not identical for comparison; the duration of the cycles shown diagrammatically has been reduced to the level of the normal cycle in graph (2). Duration of inspiration: 1 sec.; depth, 83 c.c. Duration of expiratory phase: *A* 6.2 sec., *B* 7.2 sec., *C* 8.1 sec., *D* 10.2 sec. Sustained inflation in excess of normal in *B* 0 c.c., *C* 8 c.c., *D* 20 c.c., measured from the base line of expiration *E*, *E*.

graph II (1), (2), the expiratory phase of the records is presented in a semi-diagrammatic form. The changes in the expiratory phase due to the resistance to the issue of air are well seen in graph II in which the normal

cycle and the three subjected to resistance are compared. The changes are as follows: (a) the duration of the whole cycle is increased (slower rhythm) owing to the increased length of the expiratory phase; (b) the pause is abolished, being replaced by a gradually rising line the curvature of which diminishes as the resistance increases; (c) the base line of expiration is lowered in the direction of inflation.

The results confirm our previous observation that a lowering of the base line of expiration [Hammouda and Wilson, 1932, Figs. 6, 7] by a slight resistance, external to the thorax, resisting collapse, is accompanied by a slowing of the rhythm depending on the degree to which collapse is prevented. The fact that no change in the form of the expiratory phase, the pause remaining, is to be seen in those records is due to the air outlet being unobstructed (the resistance was external to the thorax).

The explanation of the slowing is evidently that the sustained slight relative inflation by its tonic inhibitory effect diminishes the rate of discharge from that part of the central mechanism controlling the rhythm.

The intra-pulmonary pressure curve (Fig. 5 II (2)) is at its highest at the commencement when the forces of elastic recoil are at their maximum, the pressure gradually falling to the base line or until the next inspiration begins, a result which would be expected if the discharge of air were due simply to passive elastic contraction. There is no evidence here of an active muscular element in expiration such as is seen in the pressure curve of inspiration shown in Fig. 4 III (2). The form of the expiratory phase resulting from resistance to the outlet will be compared with this phase in certain human and other respiratory cycles.

Davies, Haldane and Priestley [1919] described the effects on breathing in man of resistance to inspiration and expiration (an obstruction being placed in both inlet and outlet). Owing to the type of spirometer used in their experiments, in which expiration is not recorded, the form of this phase of the cycle is not shown and it is impossible to say whether or not the base line of expiration is lowered by sustained inflation. They demonstrate a well-marked slowing of the rhythm due mainly to increase in the duration of expiration, with some increase in the duration of inspiration. This is in agreement with the observations recorded in this paper. Our observations, however, show both in anæsthetized and decerebrate animals, in which the effect of an anæsthetic is eliminated and the automatic centres may therefore be regarded as showing their normal reactions, an immediate decrease in depth of inspiration proportionate to the increase of resistance. Davies, Haldane

and Priestley find, on the other hand, an immediate and very marked increase in the depth of breathing, for example, in their Fig. 4 in the cycle immediately following introduction of a considerable resistance inspiration is doubled in depth, which they ascribe to an increase in the chemical stimulus. With a similar pressure in our experiments on animals, in which the influence of centres above the level of the mid-brain is absent, the depth would have been decreased by more than half.

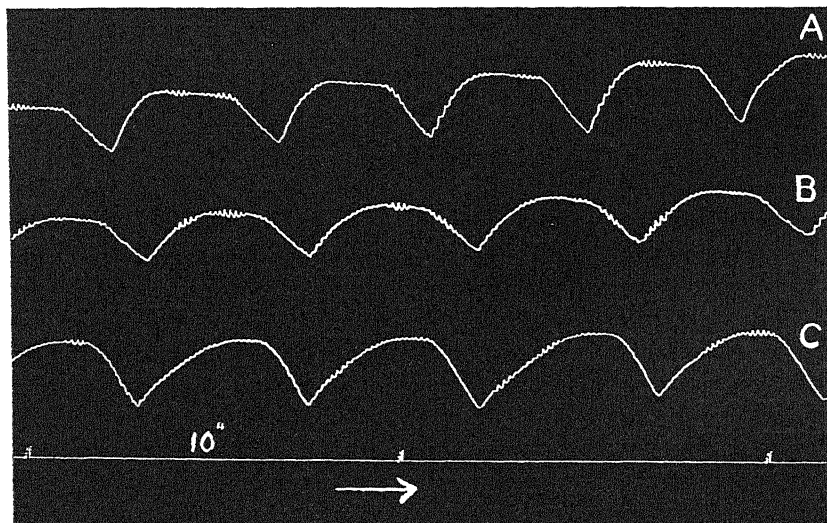


Fig. 6. Effect of anæsthesia on the cycle. Dog, 4 kg. Morphine, C. and E. No operative interference. Breathing through mask. Inspiratory and expiratory valves to 10-litre air reservoir. Bellows volume record. **A.** Fully under anæsthetic. **B.** Light. **C.** Effect of anæsthetic passing off. Rate of breathing: A 14, B 13·7, C 13 per min. Duration of cycle: A 4·3 sec., B 4·4 sec., C 4·7 sec. Duration of inspiration: A 1·15 sec., B 1·15 sec., C 1·15 sec. Duration of expiration: A 3·15 sec., B 3·25 sec., C 3·55 sec.

### 3. *Effect of anæsthetics on the cycle.*

In view of the fact that in man during sleep the expiratory phase of the cycle has been described as showing the pause, it appeared to be of interest to record the cycle of a dog breathing through a mask during recovery from anæsthesia. The anæsthetic used was C.E. No operative procedure had been carried out.

Fig. 6 shows the form of the cycle in a dog under different degrees of anæsthesia. In C when the animal was recovering from the effect of the anæsthetic but was still quiescent, the form of the cycle resembles closely



that commonly seen in man. The pause has disappeared and the record of the expiratory phase shows a characteristically curved line which will be shown later to correspond to an increased resistance to the outlet. The slight slowing of the rhythm is dependent on the lengthening of this phase and is evidently the reflex effect of the more sustained inflation. That there is no increase in the resistance to inspiration is suggested by the fact that the duration of this phase is the same in the three cases, and that the depth is not diminished but slightly increased in C.

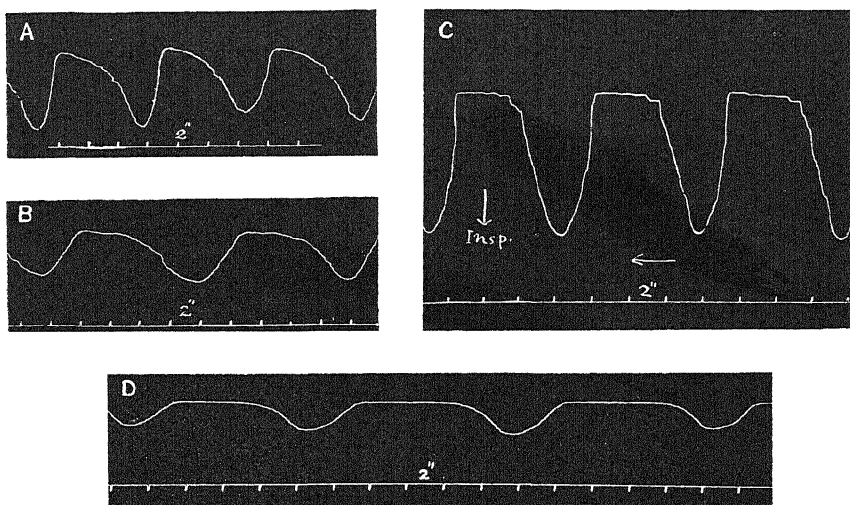


Fig. 7. Human respiratory cycles. **A.** Subject G.V.A. Stethograph record. Rate of breathing: 8.5 per min. Duration of inspiration: 1.5 sec. Duration of expiration: 5.5 sec. Ratio inspiration/expiration 1:3.6. **B.** Subject M.A.K. Stethograph. Thorax. Rate of breathing: 6 per min. Duration of inspiration: 2.8 sec. Duration of expiration: 7.2 sec. Ratio 1:3. **C.** Same subject. Stethograph. Thorax. Rate of breathing: 6 per min. Ratio 1:3. **D.** Same subject. Abdominal record. Stethograph. Rate of breathing: 4 per min. Ratio 1:3. Both subjects were adult men: awake, in an easy sitting posture. Read right to left in all curves; inspiration down, expiration up.

#### 4. Examples of human cycles.

Fig. 7 shows stethograph records from two adult male subjects, awake but quiescent. In A, B and C the stethograph was on the thorax. D is from the same subject as B and C, the record being in this case of the abdominal movements.

The breathing is slow in both subjects, in particular in K. on the occasion on which record C was taken. The cycle in A shows an expiratory

form not unusual in man (compare *l.c.* Schafer, Fig. 1). In both B, C and D a definite pause is seen, especially pronounced in C and D.

In view of the apparent difference in the form of the expiratory phase of human and other cycles in which the depth and rate of breathing vary, it appeared to be of interest to compare the expiratory phases in certain of the cycles illustrated or referred to in this paper by reducing them all to the same level of volume and duration and plotting the results in the form of curves.

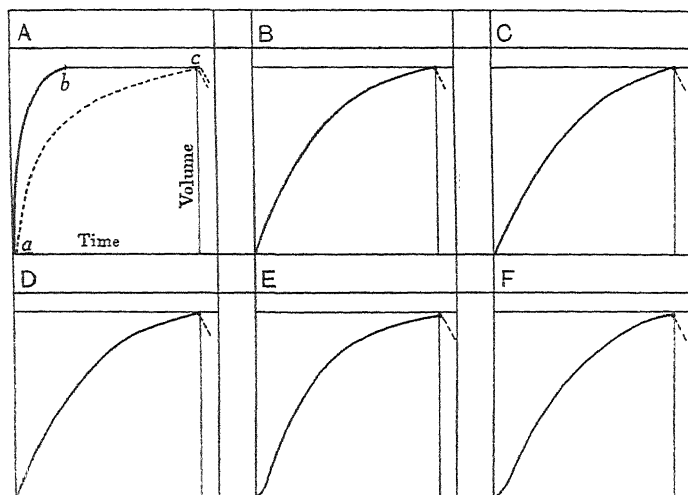


Fig. 8. Semi-diagrammatic representation of the expiratory phase of various cycles. **A.** Normal cycle. (Dog under chloralose, Fig. 5.) *a, b*, 1st phase of expiration; *b, c*, pause. Dotted line shows the form of curve *a, b* if extended for duration *a, c*. Rate of breathing:  $8\frac{1}{2}$  per min. **B.** Cycle from same figure (5) with resistance to expiration of 2.5 mm. Hg. Rate of breathing:  $6\frac{1}{2}$  per min. **C.** Cycle from Fig. 6 C. This is from the same animal as A and B before injection of chloralose or any operative procedure. The dog was recovering from C. and E. anaesthesia. Rate of breathing: 13 per min. **D.** Human cycle (Fig. 7 A). Rate of breathing:  $8\frac{1}{2}$  per min. **E.** Human cycle (from Schafer's Fig. 1, 2nd cycle, 1932). Rate of breathing: 9 per min. **F.** Human cycle (from Barcroft and Margaria, Fig. 1, VI, 1931). Rate of breathing: 21 per min.

##### 5. Comparison of the expiratory phase of various cycles.

This has been done and the resulting curves are shown in Fig. 8, A, B, C and D being from examples of respiratory cycles figured by us; E and F from cycles described by other observers.

Graph A represents the expiratory phase seen in Fig. 5 without any obstruction to the outlet with tracheal breathing; the only frictional

resistance to the exit of air from the lungs being that of the bronchioles and bronchi. The short first phase *a-b* and the prolonged pause *b-c* may be compared with that seen in the example we have given elsewhere [Hammouda and Wilson, 1932, Fig. 1, p. 84] of the normal cycle in the anaesthetized or decerebrate dog. The dotted line represents the portion *a-b*, extended to the same duration as in the other diagrams. It represents the progress of the discharge of air from the lungs by the various forces which bring this about.

Graph B represents the expiratory phase in a cycle from the same record as A but with a slight added obstruction to the tracheal outlet (Fig. 4 II, and graph C). It seems clear from a consideration of these curves that whether or not a pause occurs is dependent solely on the freedom or the reverse of the exit of air. This particular example is selected as the added resistance which abolished the pause and gave rise to this curve was sufficient to produce an initial effective pressure of 2.5 mm. Hg, which is about the same as the expiratory pressure in the human air passages in quiet breathing, namely 2-3 mm. [Starling, 1926].

Graph C is the expiratory curve from one of the cycles of a dog recovering from anaesthesia shown in Fig. 6 C. This was the same animal as A-B before administration of chloralose. The expiratory phase in A of Fig. 6 shows a well-marked pause, and if treated as the cycle in C has been would give a curve exactly similar to that shown in graph A of Fig. 8. This suggests that as consciousness is recovered the normal resistance of the upper air passages, due largely to the narrowing of the glottis in expiration, returns. This probably also explains the fact that, while in sleep in the human subject a pause in the expiratory phase is seen, this is not usually the case in the waking state.

Graph D is from the record A shown in Fig. 7, the breathing of a human subject sitting in a restful attitude.

Graph E represents the expiratory phase of a respiratory cycle of the subject whose breathing is recorded by Schafer [1932, Fig. 1].

Graph F represents similarly the expiratory phase of one of the cycles (2) in record VI of Fig. 1 of the paper by Barcroft and Margaria [1931] referred to above. This example is selected partly as it is a record of the volume expired into a spirometer and not a stethograph record of the thoracic movements as are D and E, and partly because the rate of breathing was considerably quicker (excess CO<sub>2</sub> in air), namely 21 per min., than in D or E, the other two human cycles, in which the rates were respectively about 8 in D and 9 in E.

The close resemblance of all these curves, in particular B and C (from the same animal) and D, suggests that the controlling factor upon which the shape of the curves and the absence of a pause depend is the same as in B, namely a slight peripheral resistance to the exit of air; as pointed out above this need not exceed the normal human expiratory pressure.

The form of the curves, and the fact as pointed out in discussing Fig. 4, that during expiration against a resistance the intra-tracheal pressure falls gradually from the commencement until the end of expiration, is entirely in keeping with what would be expected if the force by which air is expelled from the lungs were the recoil of an elastic system beginning at a maximum and progressively diminishing.

The observations do not give any support to the view that normal expiration is in part brought about by a muscular contraction specifically directed towards that end, in any way comparable to the active function of inspiration.

### III. STIMULI ARISING IN THE UPPER AIR PASSAGES.

It seemed possible that the form of the cycle might be influenced by reflexes arising in the glottis or other parts of the upper air passages from the movement of air over the surface or other stimuli. We propose to give here a brief account of some observations we have made on this subject.

*Method.* The trachea (dog) was opened low down in the neck and a bent glass cannula was tied in. This was connected in the usual way with valves and other apparatus for obtaining a volume record of the breathing. Into the upper end of the trachea a similar cannula was tied at about 2 cm. from the glottis. It will be seen that the upper air passages and the lungs are entirely disconnected, and therefore that any changes in the breathing which may accompany alterations in the condition of the upper air passages must be of reflex origin. A mask was in some observations attached, in others cannulae were introduced into the nostrils. A current of air or other gas can be blown through from the upper tracheal cannula producing an air current in the direction of expiration, or by sucking air from the cannula in the direction of inspiration.

No change in the form of the cycle was observed as the result of any of the procedures tried, with the exception of a decrease in depth of inspiration which will be referred to below. Currents, whether of air, oxygen or CO<sub>2</sub>, in either direction produced no change in the breathing provided there was no change of pressure.

Marked slowing of the rhythm accompanied by a decrease in depth of inspiration were, however, observed accompanying pressure changes in the air passing through the upper air passages.

An example of this is shown in Fig. 9.

It will be observed that a slight positive pressure (4 mm. Hg) affecting about 2 cm. of the upper end of the trachea, the glottis and the parts

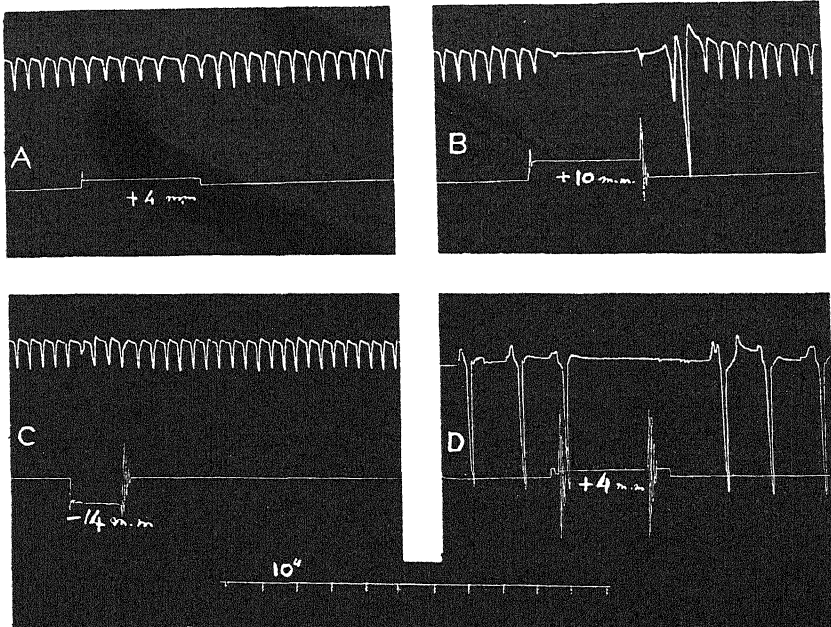


Fig. 9. Effect of pressure changes in the upper air passages. Dog. 5 kg. Decerebrate (after morphia and C. and E.). **A.** Positive pressure of 4 mm. Normal rate, 15.3; with pressure, 12 per min. Mean increase in duration of cycle with pressure, 1.15 sec. (32 p.c. increase). **B.** Positive pressure of 10 mm. **C.** Negative pressure of -14 mm. Normal rate, 18; with pressure, 16 per min. Increased duration of cycle, 0.4 sec. (11.8 p.c.). **D.** Pressure of 4 mm. Hg after vagotomy. All curves read left to right. Upper line, volume record; lower line, manometer. Inspiration, down.

above it is sufficient to produce a very marked slowing of the rhythm, the depth of inspiration being decreased from about 60 to 45 c.c. without, however, any significant increase in duration, the slowing being due to the increase in the duration of the expiratory pause. With a higher pressure (graph B) a complete inhibition of the rhythm is produced which continues for 8 sec. after the pressure has been released. Graph C shows

the effect of a negative pressure. It will be seen that while a definite slowing is produced, the effect, in view of the higher pressure, is much less marked than with a positive pressure.

It will be observed from graph D that a small positive pressure inhibits the rhythm after section of the vagi and that as in A before section this continues for some seconds after the pressure has fallen.

Attention is drawn to the fact that the pressure stimulus required to produce cessation of the rhythm is considerably less than that required before section, this may be attributed to the fact that section of the vagi lowers the excitability of the centre owing to the absence of the tonic impulses from the lungs which normally augment the responsiveness of that part of the centre controlling the rhythm. If our view be correct it is evident that a smaller stimulus will be required to inhibit the activity of this part of the centre after than before section of the vagi.

The application of a strong solution of cocaine to the upper end of the trachea, the glottis and the mucous membrane immediately above it abolishes these phenomena; it seems therefore probable that the effects are due to the stimulation of the sensory endings of the superior laryngeal nerves in the mucosa or the immediately subjacent tissues. That this is so is probable if it be remembered that Rosenthal [1862] showed that slowing or cessation of the breathing was the constant effect of exciting this nerve. We have not yet obtained experimental evidence as to whether any reflex effects on the breathing occur arising in the upper air passages after section of the superior laryngeal or glossopharyngeal nerves. We cannot therefore at present state precisely the area from which these stimuli arise.

The reflex slowing described as the result of pressure change in the upper air passages may in part account for the very marked decrease in the rate of breathing recorded by Haldane and his co-workers, referred to above, in the human subject; it cannot, however, explain the slowing obtained from resistance to expiration illustrated in Fig. 5 of this paper, but may account for the slowing seen in some cases of mask breathing in the dog, and that seen in Fig. 6.

The similarity of the phenomena described above to the effects of expansion of the lungs suggests that they form a part of the same reflex function, and that while the origin of the former is probably in the terminations of the superior laryngeal in the mucosa of the larynx and upper third of the trachea, that of the latter may be in the terminations of the pulmonary vagi in the mucosa of the bronchi and bronchioles.

No stimuli to the upper air passages which we have yet employed have produced the characteristic acceleration of the rhythm which accompanies deflation of the lungs.

#### DISCUSSION.

Our former investigations on the effects of inflation and deflation of the lungs demonstrated that two influences reached the centre from the lungs; the one inhibitory of inspiration, the other augmentor of the rate of the rhythm. In that investigation no evidence was found of any reflex controlling expiration or the form of that phase of the cycle, with the exception of the slowly developing increase in the tonus of the expiratory muscles resulting from sustained and considerable expansion of the lungs, which might even develop into a reversal of the normal process, expiration becoming the active, inspiration the passive phase. The reflex production of an increased expiratory tonus has no counterpart in the normal cycle even in the slow rhythm of slight inflation. It is shown after vagotomy, and appears to correspond to the reflex effects described by Fleisch which he regards as evidence of the proprioceptive reflexes arising in the muscles of inspiration and reacting on the expiratory phase. That proprioceptive reflex effects arise as in other skeletal muscles is probable. As pointed out in the introduction to this paper the abdominal muscles do not show any evidence of their presence, and the form of the expiratory phase with tracheal breathing in the decerebrate animal, even in hyperpnœa, indicates either that these reflexes have little influence on the expulsion of air from the lungs or that the changes of tone in the internal intercostals are counterbalanced by corresponding changes in the opposite direction in the external intercostals.

The observations recorded in this paper appear to explain the difference in the form of the expiratory cycle observed in dogs with tracheal breathing, and sometimes in man, and the common human type.

It is, however, evident that something more is required to explain the fact that with very varying rate of breathing in the conscious human subject and probably in animals in ordinary conditions of active life, the diminishing volume of the lungs reaches the base line of expiration immediately before inspiration begins without any intervening pause.

This is well seen in Barcroft and Margaria's figures [1931] of breathing accelerated by either excess of  $\text{CO}_2$  or by effort. In the decerebrate dog the only marked change under similar conditions ( $\text{CO}_2$ ) is a progressive shortening of the pause, the duration of the inspiratory phase and the first phase of expiration undergoing little change whatever

the depth. In Margaria and Barcroft's observations, while there is slight diminution in the duration of inspiration, the shortening of the cycle with increased rate is mainly at the expense of the expiratory phase.

Our observations provide no evidence that the rate of discharge of air from the lungs is regulated by any action of the expiratory muscles. Were this so its effect should be seen in the cycle of breathing in decerebrate animals in which such reflexes as may occur would not be suppressed by the action of anæsthetics. The evidence is, in fact, entirely in favour of the control of expiration being effected by a mechanism regulating the frictional resistance at the exit. The increase in the dead space in dyspnoic conditions suggests that there is some reflex relaxation of the bronchioles in rapid or deep breathing in addition to the automatic dilatation of the air passages facilitating both ingress and egress of air which accompanies any increase in the volume of the lungs. The slight change in the duration of either the inspiratory or expiratory phase which occurs in dyspnoea in the tracheal cycle of decerebrate animals excludes the possibility of the regulating mechanism acting within the lungs. The regulation of the rate of discharge of air must therefore be through the accessory muscles of respiration in the upper air passages. The exact adjustment of the aperture of the glottis and the external nares to the depth and rate of inspiration is well known, in some animals such as the horse this reflex is essential to the proper adjustment of respiration in muscular exertion; even in man, where mouth breathing is normal under such conditions, it is of some importance.

The usual form of the expiratory phase of the respiratory cycle in man implies that this reflex control extends also to the aperture of the outlet during expiration, the diminished frictional resistance with deep breathing cannot be regarded as merely the passive effect of the expansion during inspiration. The extent of this regulation of the exit and how it is effected is not so evident.

Hess [1930], from observations of the position of the dome of the diaphragm with the open thorax, has observed changes in the tone of that muscle increasing progressively with decreasing volume of the lungs. He regards these effects of collapse of the lungs and thoracic walls as regulatory of the process of expiration. He states also that reflex effects similar to those produced by inflation and deflation of the lungs can be elicited by passive movements of the thoracic walls without change in the lung volume, suggesting that the nervous control of the breathing is not entirely dependent on impulses received by the centre through the pulmonary vagi. Creed and Hertz [1933] have recently produced evi-



dence which they believe confirms the results of Hering and Breuer and Head, using the latter author's method of the diaphragmatic slip, in rabbits, that contraction of the diaphragm is the normal response to deflation. We have never observed any indication of such a reaction to deflation of the lungs on the volume record with the intact thorax. The conditions, which we are at present investigating, under which such tonic contractions of the diaphragm occur appear to be rather complex. A study of Creed and Hertz's figures indicates, as was originally pointed out by Schenk [1903], that this response to deflation does not occur unless the collapse of the lungs is sudden and complete; there does not seem to be, as suggested by Hess, an increase of tone increasing progressively with the diminution in the lung volume.

The regulation of the frictional resistance to the outflow of air through the glottis and external nares might be effected either by a control of the rate of relaxation of the abductor muscles (and dilator of nares) or of the contraction of the adductors. The sustained dilatation of the nares in hyperpnœa, in animals in which this function is important, suggests that the former is the more probable. This reflex control of the resistance in the upper air passages to the inlet and outlet of air seems to be absent in anæsthetized and decerebrate animals, suggesting that it is related to the activity of centres above the level of the mid-brain.

We hope by a modification of the method described for examining the reflex influence of stimuli arising in the upper air passages to further investigate this question.

#### SUMMARY.

1. The presence of the upper air passages in anæsthetized or decerebrate animals diminishes the depth of inspiration and prolongs the first phase of expiration, as compared to tracheal breathing.
2. Resistance to inspiration is shown to cause a decrease in depth and an increase in the duration of the inspiratory phase of the cycle.
3. The expiratory pause is shown to occur in the normal human cycle under certain conditions.
4. Resistance to expiration about equal to that normally seen in the air passages during that act abolishes the expiratory pause, the presence or absence of which is therefore dependent on the extent of the frictional resistance to the exit of air offered by the upper air passages.
5. The form of the expiratory phase of the respiratory cycle usually seen in man is shown, by comparison with the effects of air resistance to the air exit in tracheal breathing in animals, to be due to the resistance

to the exit of air through the upper air passages which must vary with the depth and rate of breathing and is, it is suggested, under some reflex nervous control.

6. A method is described for the investigation of the reflex effect of stimuli arising in the upper air passages. A reflex to positive pressures is shown to occur which is similar to that produced by expansion of the lungs.

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## THE SUBMAXIMAL RESPONSES OF THE SINGLE MUSCLE FIBRE.

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It has been shown by the writer [Gelfan, 1930, 1931], and also by Pratt [1930], that in the normal skeletal muscle stimulation of single fibres with micro-electrodes gives rise to submaximal contractions, graded according to the strength of the stimulus. The same results were obtained by Brown and Sichel [1930], and Asmussen [1932] on single fibres that had been dissected out. The following experiments were undertaken in order to determine whether these submaximal contractions differed in any way in their mechanical response from the maximal, all-or-none twitch, which results from stimulation with electrodes of larger area. It has already been shown by Gelfan and Bishop [1932] that the submaximal responses do not have action potentials, whereas in the identical fibre a characteristic diphasic action potential is obtained when it is stimulated to contract maximally. This is in harmony with the observations of Gelfan and Gerard [1930], and Hashida [1931] that the graded responses are not propagated throughout the length of the single fibre, and that the progressive spread of the localized response, as the strength of the stimulus is increased, is continuous only to a limited extent, after which the maximally conducted response is evoked. The maximal response is always accompanied by an action potential, whereas the graded submaximal contractions are not. These observations indicate not only that action potential and contraction are not inseparable, but that in any single striated fibre the former is of the explosive all-or-none type of mechanism whereas the latter is not. It has therefore been suggested by the author [1931], that when the muscle fibre responds maximally, whether it is stimulated indirectly or directly (electrically), it does so by virtue of the operation of the action potential mechanism.

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That the contractile mechanism might be directly stimulated without at the same time initiating the "excitatory change" was already recognized by Lucas [1907, p. 273]. He considered this possibility when he found that the local cathodic contraction, in contrast to the propagated response, was independent of the current gradient. Contractures also do not have wave-like action potentials [Gasser, 1930; Bremer, 1932]. The submaximal contractions as obtained by microstimulation might of course also be considered as of the nature of a contracture or local cathodic contraction. The latter, however, are characterized by the fact that they persist as long as the stimulating current is flowing. In the experiments here reported, therefore, records were obtained of the contraction curves of the submaximal and maximal responses of single fibres in order to compare their time relations, the stimuli being either induction shocks or constant currents of long duration.

#### METHOD.

The frog's (*Rana temporaria*) excised sartorius was used for all of the experiments here reported, prepared with a sufficient amount of tissue from the opposite leg, by means of which it could be suitably pinned on the glass slide provided with a piece of cork cemented to it. The tendon at the tibial end was tied with a thread which supported a small weight. The preparation under the microscope could be satisfactorily viewed by transmitted light, and any single fibre on the surface of the muscle could be clearly made out. The muscle was surrounded with cotton wool soaked with Ringer's solution, and was frequently irrigated with fresh solution.

The electrodes used were similar to those previously used by the author. Either platinum wire drawn out inside quartz capillaries to a few  $\mu$  in diameter, or quartz capillaries of the same dimensions filled with frog's Ringer solution were used. The latter type, before filling with the Ringer's solution, are cemented into glass tubes of suitable dimensions. The glass tubing is then filled with Ringer's fluid and the latter is forced into the capillary to the very tip by means of pressure. An Ag-AgCl coil is then placed into the glass tube. The electrode is moved and oriented under the microscope by means of a Chambers' micromanipulator. Only one micro-electrode was used directly in contact with the fibre, the other indifferent electrode being an Ag-AgCl coil about 4 cm. long, placed near the muscle and covered by the same cotton wool that surrounded the muscle. The total diameter of the micro-electrode being only a few  $\mu$  in diameter, it is possible to observe and record as near the point of stimulation as desirable.

The method of recording was essentially that of Pratt and Eisenberger [1919]. The preparation was sprayed with minute mercury droplets which were illuminated by a bright source of light from the side. The point of light that is reflected through the microscope by one of the mercury droplets on the responding fibre passes through a horizontal side eyepiece, and then to the motor-driven camera 1 ft. away from the microscope. The side eyepiece is so adjusted that the point of light from the mercury droplet is in focus on the camera film when the fibre is in focus for the observer. While the mercury globule is illuminated when recording, the responding fibre is also continuously observed with transmitted illumination.

Although the muscle was weighted by about 3 g. only, the contractions took place under fairly isometric conditions, since a single fibre had to contract against the resistance of the 400 fibres of the sartorius. No movement can be detected without magnification when only one fibre is responding.

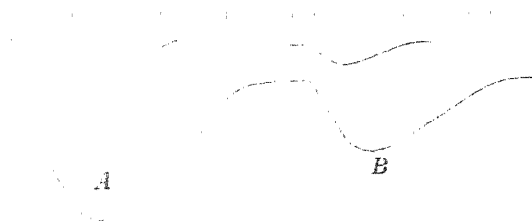
## RESULTS.

### *Responses to single induced shocks.*

The essential condition for obtaining submaximal contractions in the single uninjured muscle fibre, as has already been pointed out [Gelfan, 1930; Gelfan and Gerard, 1930], is that the electrode should be of sufficiently small size. Asmussen [1932] obtained graded responses in the single fibre without the use of micro-electrodes. But in his experiments a single fibre was dissected out, and such an operation is scarcely possible without some injury to the fibre. Graded responses can be obtained in the injured fibre with diffuse stimulation [Hintner, 1930], and also in hearts that are in poor condition [Carlson, 1906]. In the curarized preparation, as already shown by the writer [1931], microstimulation is also necessary in order to obtain submaximal responses. This point is of some importance since Asmussen [1932, 1933], accepting the views of Lindhard [1932], considers the all-or-none behaviour of the fibre as a property of the end-plate. If this were true, microstimulation would not be necessary in the curarized preparation in order to obtain graded responses in the single fibre. Curarized preparations were at first used in the present experiments but later abandoned, as no advantage was found in the curarized over the non-curarized muscle. Not only do the micro-electrodes permit very fine localization of the stimulus, but they were also applied at the extreme pelvic (nerve-free) end of the muscle.

Plate I is a continuous record showing graded contraction curves of a single fibre. The stimulus was graded by movements of the secondary

coil. The current in the primary was delivered at intervals by a 'metronome' interrupter, with condensers across the mercury cups to eliminate sparking. The duration of the submaximal responses, as may be observed from the record, is no longer than that of the maximal (last two responses in the record). Indeed the smaller responses are actually of shorter duration, approaching that of the maximal twitch as the magnitude of the mechanical response approaches that of the entire fibre. If the time relations of the contractile process at any point on the fibre are the same in a submaximal as in a maximal contraction, the longer



Text-fig. 1. A. Camera lucida drawings of a maximal and submaximal twitch of a single fibre stimulated by induced shocks, in which the shape and form of the contraction curves and time relations may be compared. Taken from Plate I, magnified  $\times 4$ ; total magnification  $\times 700$ . B represents the same for the responses to constant current stimuli of 0.6 sec. duration taken from Text-fig. 3. Time signal above 0.01 sec. The record of the responses to the induced shocks was chosen in this case because of the unusual magnitude of the mechanical response of a single fibre. The other records of responses to induced shocks are more of the order of magnitude as represented by the responses to constant current.

duration of the maximal responses observed is not surprising since the activity is conducted over the entire length of the fibre, whereas in the submaximal response only a fraction of the fibre is involved. Apart from this difference in duration, the shape and form of the maximal and submaximal curves, as may be observed in Text-fig. 1, are essentially the same. The duration and magnitude of the response may vary somewhat in different experiments, depending upon the condition that the preparation is in, length of the muscle, mechanical conditions during any given experiment, etc. But whatever the duration, or shape and form of the maximal response may be under any given experimental condition, the relation between any given fraction of the maximal mechanical response and the maximal response is always of the kind shown in Text-fig. 1.

*Responses to constant current.*

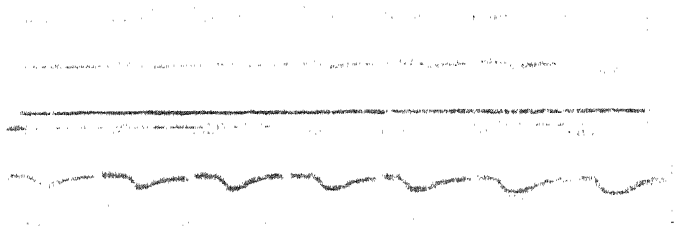
The responses of a skeletal muscle fibre to constant current, as Biedermann [1895, p. 160] demonstrated, may be of three types: a simple twitch, a twitch followed by a persistent shortening, and a persistent contraction not preceded by the twitch. The local cathodic contraction, as figured by Lucas [1908], which lasts as long as the exciting current does, is obviously the same as the third type mentioned by Biedermann. Reversible contracture type of responses to direct and indirect electrical stimulation have also been described by Bremer [1932]. Although the graded responses of the single fibre to induced shocks, as shown above, have time relations no longer than that of a twitch, it might still be argued that the submaximal responses are all examples of the local cathodic contraction and that the twitch-like brevity is permitted by the briefness of the stimulating current. As will be seen below, however, the graded contractions of the single fibre when stimulated by constant currents of long duration can be of any of three types described by Biedermann.

To avoid confusion, it is necessary first to point out the apparent reversal of polar effects with constant currents when micro-electrodes are used. The electrode system consisted of one micro and one large indifferent electrode. With this arrangement there is an apparent reversal of Pflüger's law, for the response occurs at the closure of the current when the micro-electrode is anode, and at the opening when it is cathode. The explanation of this phenomenon probably lies in the well-known fact that a localized electrode applied to a tissue induces an electric pole of opposite sign in the neighbouring parts of the tissue, and it would seem that in the conditions described this secondary pole is more effective than the primary.

Of the three types of response to constant current mentioned above, the twitch is of greatest interest in this case. The occurrence of submaximal twitches definitely demonstrates that in the single fibre graded contractions of twitch-like duration may be obtained with constant currents that far outlast the duration of the response. Plate II shows a series of responses graded by increasing the strength of a constant current by means of a potentiometer in the circuit. The micro-electrode in this case is the anode and the contractions are only at the make of the current. The duration of the responses is of the order of that of a twitch, although the stimulus is of about 0.5 sec. duration. Similarly, Text-fig. 2 shows responses at cathode to the break of the current. The last responses in

both cases are maximal, as further increase in stimulus strength did not increase the height of the mechanical response (sufficient increase in stimulus strength with consequent spread of current evokes additional units to respond maximally with the well-known step-like gradation). The shape and form of the submaximal contractions are not significantly different from the maximal ones, except for the magnitude of the response. As the camera lucida enlargements in Text-fig. 1 demonstrate more clearly, the duration of the submaximal response is actually shorter than the maximal twitch.

The response at the break of the current can be graded not only by the variation of the current intensity but also by grading the duration of the

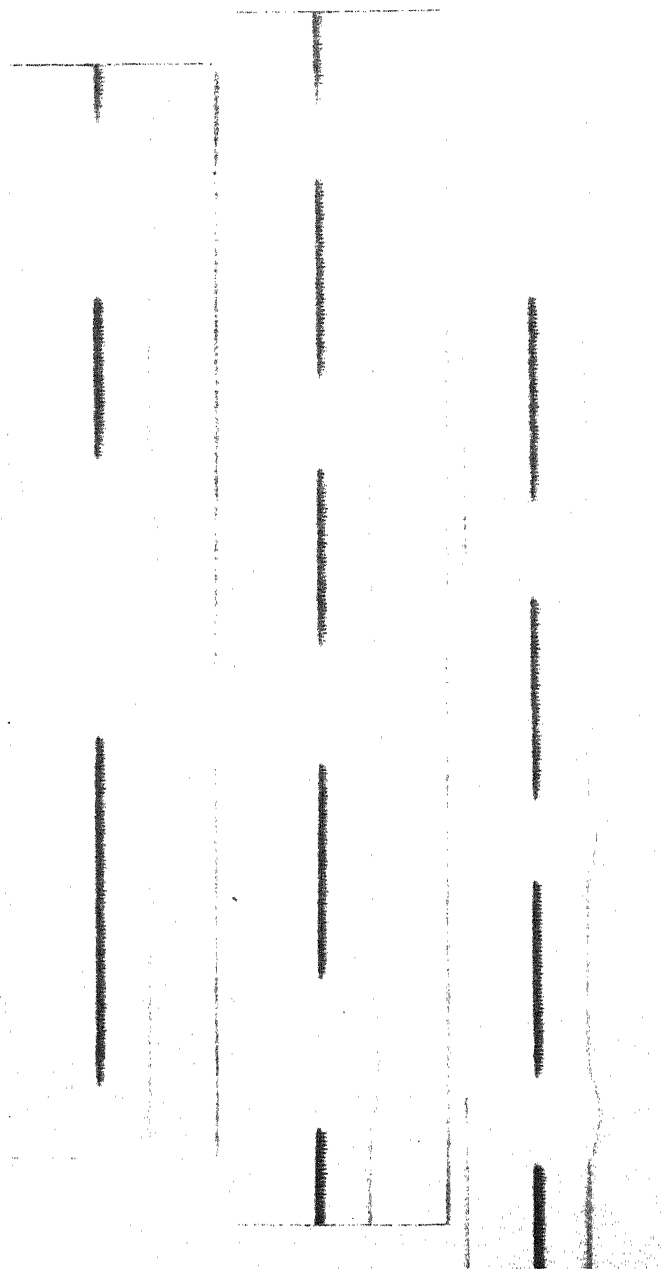


Text-fig. 2. Graded responses of twitch-like duration to break of constant current at cathode. To be read from left to right and from above downward. Non-polarizable electrode  $10\mu$  in diameter. Stimuli, lasting 0.6 sec., and graded by potentiometer, were delivered every 2 sec. The end of the time marker (0.01 sec.) above myogram in each strip of the record represents the break of the current. Most of the record between each make and break has been cut out, each strip representing only a small fraction of the entire duration of the stimulus. Operation of switch as in Text-fig. 2. Temp.  $17^{\circ}\text{C}$ . Microscopic magnification  $\times 170$ .

stimulus. With a current duration of about 1 sec., and an intensity that is maximal for this duration for the single fibre, fractions of the maximal response may be obtained by decreasing the duration and keeping the intensity constant. This is true for both the twitch-like responses and the sustained contractions. The latter effect may be seen in Text-fig. 3. A similar observation on the effect of duration of a tetanizing current of constant intensity giving sustained effects has already been observed by the author [1930].

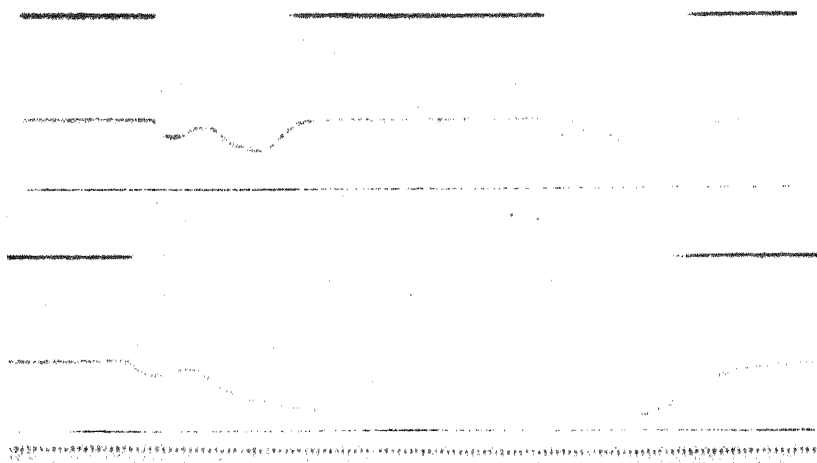
The galvanic contractures in the single fibre were obtained both at the make of the current when the micro-electrode was the anode, in which case the response lasted as long as the current was on, and at the break of the current when the micro-electrode was the cathode. In the latter case, persistent contractions, some of which lasted several seconds, were





Text-fig. 3. Continuous record of graded contracture responses of a single fibre to break of constant current at cathode. Non-polarizable electrode  $15\mu$  in diameter. Read from left to right, from above downward. Time marker (0.01 sec.) indicates duration of stimulus, make at left or beginning of signal, and break at end of signal. Demonstration of inhibiting effect of make of current on contraction produced by the break at cathode. Temp.  $16^{\circ}\text{C}$ . Microscopic magnification  $\times 170$ .

obtained and these always began only after the cessation of the current. Text-fig. 3 shows graded contractures of the single fibre elicited by the break of the current at the cathode. The active inhibitory effect of the make of the current after the contracture has started as a result of the break of the current is well illustrated here, and also in Text-fig. 4. The inhibitory effect of the current on a muscle in a state of excitation was long ago demonstrated by Biedermann [1895] on the heart and on the veratrinized sartorius. Recently it has again been demonstrated by



Text-fig. 4. Persistent maximal contraction preceded by twitch at break of current, and inhibition by the make. Cathode  $15\mu$  in diameter. Only one fibre responding, showing greater degree of shortening of maximal contracture as compared to maximal twitch. End of signal above myogram is break of current, beginning is the make. Temp.  $16^{\circ}\text{C}$ . Time below 0.01 sec.

Bremer [1932]. In the experiments of the last two investigators it is the anode make that exerts the inhibitory effect, whereas in our case, because of the reversal of polar effects with the micro-electrode, the effect is at the cathode make.

Text-fig. 4 shows the contracture response preceded by a twitch. Only one fibre is responding, but the twitch in this case is already maximal. The response is at the break of the current at the cathode. In the upper portion of the record the contracture after the twitch is inhibited before its full development by the making of the current again, shortly after the

response began. In the lower portion of the record, however, where the fuller development of the persistent contraction is permitted before the current is made again, the very much greater shortening may be observed. Visually, the maximal contracture response to the break of the current at the cathode is strikingly similar to a tetanus or to the type of response evoked by pricking a muscle fibre of the retrolingual membrane, as reported by Gelfan and Bishop [1933].

#### DISCUSSION.

It is clear that submaximal contractions may be obtained in the single fibre that, in their time relations, are in no way different from a twitch. This is equally true for constant current stimuli as it is for induced shocks. Furthermore, it is possible to differentiate, in the submaximal responses of the single fibre as well as in the all-or-none responses of the fibres as a whole, contracture from twitches. The graded twitch-like responses in the single fibre, therefore, cannot be regarded simply as local cathodic contractions, as Ritchie [1932] suggests. The submaximal twitches, however, do differ from the maximal ones at least in one important respect. They are not accompanied by an action potential [Gelfan and Bishop, 1932]<sup>1</sup>. Also, they are not propagated throughout the length of the fibre, and need a stimulus of restricted area.

The question as to the validity of the all-or-none principle has now become mostly a matter of definition. There is an all-or-nothing relation between stimulus and electric response in the muscle, and presumably between the stimulus and the propagated disturbance, but the contractile process is fundamentally capable of continuous gradation. This further indicates that contraction and electric response are distinct mechanisms. The fact that the two responses begin simultaneously [Roos, 1932] does not preclude their separability.

<sup>1</sup> It is difficult to accept the claim of Henriques and Lindhard [1920] and Lindhard [1932] that a muscle stimulated directly (by large electrodes, so that many or all fibres are contracting maximally) does not have an action potential. Action potentials have frequently been measured from muscles completely curarized, in which case there was no question of stimulating intramuscular nerve fibres. Adrian and Owen [1921] were able to demonstrate action potentials in the denervated gastrocnemius and sartorius. Henriques and Lindhard [1923] claimed that the action potentials in the last experiments were due to stimulus escape, but Adrian [1925] published further records from denervated muscle in which the form of the response was shown to be quite different from that of the stimulus escape. Records can easily be obtained with the Matthews's oscillograph showing both the stimulus escape and action potential, the former some distance from the latter, from a completely curarized sartorius. Furthermore, spontaneous discharges from a muscle in NaCl can be measured when the preparation is curarized [Adrian and Gelfan, 1933]. There is no question of stimulus escape in this case.

All the events that can occur when a muscle fibre is adequately stimulated are not interdependent to the degree that they must either all occur or not at all. Whatever the structural basis of the various processes may be, it is obvious that the fibre can be stimulated to give a slow response (reversible contractures) or a rapid one (twitch), and that the electric response may or may not accompany the mechanical one. Furthermore, the different processes involved do not necessarily obey the same laws. The all-or-nothing behaviour which characterizes the action potential mechanism is not a fundamental property of the contractile process, as is indicated by the various types of contracture and by the submaximal responses of the single fibre.

#### SUMMARY.

Records have been made of the contractions of single muscle fibres of the frog's sartorius which permit analysis of the duration and shape and form of the mechanical response to induction shocks and constant current of long duration. The graded submaximal responses in the single fibre could thus be compared to the maximal ones in the same fibre.

All of the three types of responses that can be produced in the muscle as a whole by diffuse stimulation with constant current can also be duplicated in the submaximal responses of the single fibre. The rapid submaximal responses to constant current in the single fibre have the time relations of a twitch.

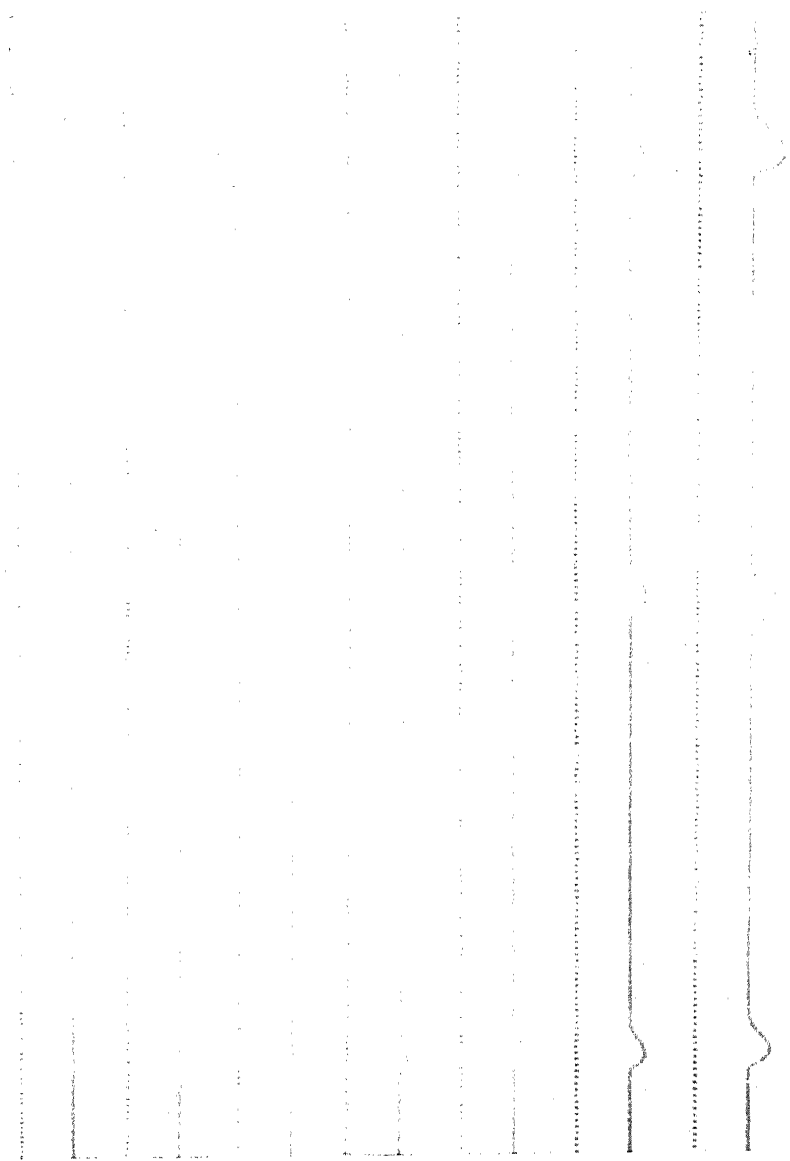
There is an apparent reversal of polar effects when a micro-electrode and a large indifferent electrode are used for stimulating.

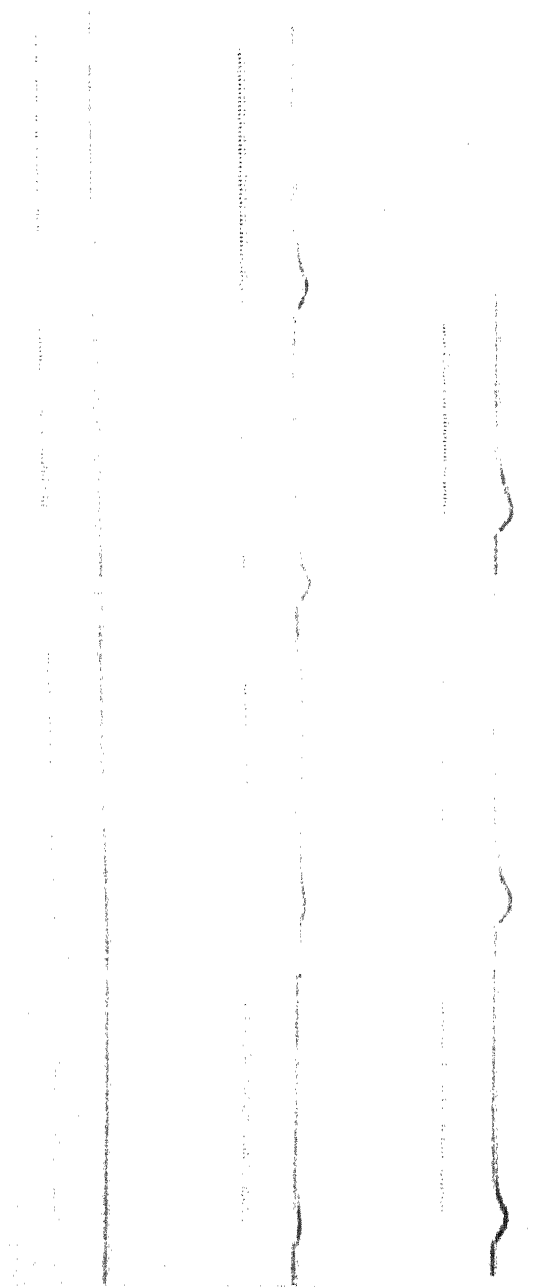
A contracture produced by the break at the cathode can be actively inhibited by the make of the current again.

I wish to acknowledge my indebtedness to Profs. E. D. Adrian and J. Barcroft for all the courtesies extended to me during my stay at this laboratory as a Guggenheim Memorial Fellow.

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## EXPLANATION OF PLATES I AND II.

### PLATE I.

Continuous record showing graded responses of a single fibre in the sartorius muscle to single induction shocks, increasing progressively in strength. Record reads from left to right and from top to bottom. Last two contractions are maximal. Time signal at top of record 0.01 sec. Unipolar stimulation with non-polarizable electrode  $5\mu$  in diameter (see also text). Microscopic magnification  $\times 170$ . Temp.  $16^{\circ}\text{C}$ .

### PLATE II.

Graded responses of twitch-like duration to constant current stimuli. To be read from left to right and from top to bottom. The non-polarizable electrode,  $15\mu$  in diameter, was the anode, and responses are only at the make. Stimuli lasting 0.5 or more seconds were graded by potentiometer and delivered every 2 sec. Temp.  $16^{\circ}\text{C}$ . Time 0.01 sec. Microscopic magnification  $\times 170$ . The beginning of the time signal at the left in each case indicates the make of the current, and the end on the right the break of the current. A hand key of a double-pole mercury cup switch made and broke contact of both the stimulating current and the current illuminating mirror on vibrating tuning fork. The imperfect relation between beginning of stimulus and beginning of time marker is due to the fact that the contacts in the two mercury cups were not made perfectly simultaneously.

THE BEHAVIOUR OF THE LIVER GLYCOGEN  
DURING DECEREBRATION HYPERGLYCÆMIA  
AND THE INFLUENCE OF ATROPINE AND OF  
ERGOTAMINE ON THIS CONDITION.

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(*Received September 27, 1933.*)

IN the experiments of Donhoffer and Macleod [1932] on decerebration hyperglycæmia, it was shown that atropine injected in repeated doses of 1 mg. was capable of preventing or at least retarding the rise in the blood sugar percentage in fasted rabbits in which the vagus nerves were cut. In well-fed animals with large stores of liver glycogen, on the other hand, atropine and vagotomy had no influence on the development of the hyperglycæmia. In these experiments the influence of ergotamine was undecisive because of difficulties in the collection of blood from the ear vein. Since it is important in solving the problem of the nature of the nerve control of the blood sugar level to know exactly the effect of these two drugs on decerebration hyperglycæmia, it was considered advisable to reinvestigate their effects with the difference that the vagus nerves are left intact. The results of such experiments are shown in Tables I and II. In another series of experiments, Donhoffer and Macleod found that the degree of hyperglycæmia following decerebration was not related to the percentage amount of glycogen present in the liver to start with. They also found that the amount of sugar arising from the glycogen which disappeared from the liver during the course of a decerebration experiment on a previously fasted animal was far less than that required to cause a comparable increase in the blood sugar percentage when given by continuous intravenous injection to rabbits under amytal. This led to the conclusion that the nerve disturbance set up by the decerebration must excite a new formation of sugar in the liver—a secretion, as Claude

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Bernard thought—and that the glycogen found present in the organ was merely a by-product of this process which might either become increased or decreased in amount during an experiment. Even supposing that over-production of sugar in the liver, rather than decreased utilization in the tissues, is the cause of the hyperglycæmia which follows decerebration, it is obvious that only a very approximate estimate of the amount of extra sugar, which would be required to produce it, could be obtained by seeing how much glucose had to be administered by continuous intravenous injection into a normal animal to bring about a corresponding rise in the blood sugar level. Quite apart from a possible effect of anæsthesia, differences in the two animals—the decerebrated and the anæsthetized—might depend on the state of carbohydrate metabolism at the time of the experiment; whether the muscles, for example, were building up glycogen or breaking it down. Such comparisons would only be justifiable when changes in the glucose, glycogen and lactic acid content of the entire body were taken into account, and even then one would have to be certain that the same amounts of carbohydrate were being metabolized by the two animals under comparison.

Although there is, therefore, no way of knowing how much extra sugar is necessary to cause a degree of hyperglycæmia comparable with that following decerebration, it was considered important to ascertain how the percentage of liver glycogen would behave from hour to hour when large amounts were present at the time of decerebration. If any degree of parallelism could be detected between the rate at which the percentage of sugar rises in the blood and that at which the glycogen percentage falls in the liver, it would be strong presumptive evidence that over-production of glucose out of preformed glycogen in this organ is mainly responsible for the hyperglycæmia which follows decerebration in well-fed animals. It is quite certain that extra sugar production out of preformed glycogen in the liver is at best only a secondary factor in causing decerebration hyperglycæmia in fasted animals, but this does not rule out the possibility that this is the main source of the sugar in well-fed ones with much stored glycogen in the liver. This increased glycogenolysis might conceivably last until all the preformed glycogen has been used up and then be followed by increased gluconeogenesis.

The methods used for decerebration, the removal of material for analysis and the determination of glycogen, blood sugar, etc. were the same as those described by Donhoffer and Macleod. The only experimental detail that requires mention here concerns the choice of animal and its feeding prior to fasting. From various laboratories, as well as this

TABLE I. Effect of different amounts of atropine on the percentages of blood sugar and liver glycogen after pontine decerebration.

Exp. No.	Wt. of rabbit	venous injections of atropine (mg. per kg., †)	Blood sugar (mg. per 100 c.c. blood)												Liver glycogen	
			Time after decerebration (hours)												Initial	Final
			1/2	1	1 1/2	2	2 1/2	3	3 1/2	4	4 1/2	5	5 1/2	6		
16	1.9*	(1) 1.05 (2) 1.05 (3) 1.05	130	160	180	190	225	240	255	295 (1)	325	390 (2)	440 (3)	—	6.9	2.8
18	1.3	(1) 2.4	225	—	190	145	200	250	345	435 (1)	410	390	375	365	340	—
20	0.8	(1) 5.3 (2) 10.6	155	—	225	270	280 (1)	—	335	395	480	410 (2)	365	375	390	12.6
22	1.4	(1) 4.5 (2) 4.5	145	—	210	210	165	170	—	280 (1)	245	250	220	210 (2)	200	0.5
24	0.9	(1) 10	180 (1)	155	145	135	165	175	175	390	265	265	290	450	—	10.9
26*	0.8	(1) 11	145	—	170	220	225	230	230 (1)	340	300	325	330	350	380	—
27*	1.3	(1) 6	250 (1)	250	160	230	205	205	215	290	260	305	340	—	—	0.6

\* Animals fed high carbohydrate diet and not fasted, except Nos. 26 and 27, which were fasted overnight.

† The injections were made at the time intervals indicated by the numbers. The amount of atropine given at each injection is also given in this column.

one, the results of recent years have shown that the behaviour of carbohydrate metabolism under any particular condition, such as administration of insulin or adrenaline, fasting and even such violent disturbances as those caused by decerebration, may be influenced by the age of the animal, the season of year, the time of day, as well as by the previous feeding. In order to obtain consistent results even in decerebration experiments, it is therefore necessary that the experimental conditions be rigidly standardized. This has been done as far as possible in the present investigation, and would have been done completely had a suitable supply of rabbits been available.

(1) *The effect of atropine.* (Table I.)

In one of the animals (No. 27) the liver glycogen to start with was low, as a result of previous fasting, and the atropine given at the time of decerebration retarded although it did not prevent the development of hyperglycæmia. There is no doubt, however, from the results of Donhoffer and Macleod, that atropine under these conditions does usually prevent pontine decerebration from having any effect on the blood sugar level. On the other hand, when the atropine is given to carbohydrate-fed animals having large amounts of liver glycogen, it does not retard the rise in the blood sugar level following decerebration unless the dose be excessive (10 mg.), and even then the retardation is only temporary. In Exps. 20 and 22, in which atropine was given after the hyperglycæmia had become established, only a doubtful depression followed in the blood sugar level, and in Exp. 26, in which as much as 11 mg. was injected, the hyperglycæmia was not affected. On the other hand, when a large dose of atropine (10 mg.) was given at the same time as decerebration was performed, as in Exp. 24, it did distinctly retard the development of the hyperglycæmia. Doses of atropine less than 5 mg. had no effect, as is seen in Exps. 16 and 18.

As might be expected, the atropine did not influence the break-down of liver glycogen.

(2) *The effect of ergotamine.* (Table II.)

In the experiments of Donhoffer and Macleod the results obtained after injecting ergotamine were not conclusive, because of the difficulty of collecting blood from the ear veins. It was decided to repeat these experiments by collecting the blood from the carotid artery. Stock-fed animals

TABLE II. Behaviour of the percentages of sugar and lactic acid in the blood, and of glycogen in the liver after intravenous administration of ergotamine alone and of ergotamine plus atropine and vagotomy following decerebration.

Exp. No.	Wt. of rabbit (kg.)	Region of decerebration	Injection amount (mg. per kg.) <sup>†</sup>	Blood (mg. per 100 c.c.)					Time after decerebration (hours)				Liver glycogen p.c.	
				Constituent determined	Initial value	1	1½	2	2½	3	3½	4	Initial	Final
57	1.3	II	(b) 0.4	Sugar	185	120	140	115	128	135	140	165	4.0	1.8
				L. acid	67			121				116		
58	1.0	III	(b) 0.5	Sugar	138	174	174	165	178	153	163	203	4.3	3.3
				L. acid	45			64				62		
61	1.7	III	(b) 0.5	Sugar	143	115	125	138	130	125	140	125	2.7	3.2
				L. acid	20			30				40		
63	1.9	III	(b) 0.5	Sugar	163	158	163	205	228	205	190	155	2.3	0.8
				L. acid	36			202				87		
64	1.3	III	(b) 0.8	Sugar	135	110	105	130	120	125	155	225	0.8	0.8
				L. acid	30			30				39		
65	1.1	III	(b) 0.9	Sugar	125	150	120	150	135	130	140	180	3.6	3.9
				L. acid	84				33			37		
51	1.1†	III	(a) 10 (b) 0.5	Sugar	165	180	130	120	105	105	140	170	5.5	3.0
				L. acid	32			93				93		
54	1.6†	III	(a) 10 (b) 0.6	Sugar	203	235	197	151	183	151	193	193	2.2	3.8
				L. acid	—			64				95		
56	1.8	III	(a) 10 (b) 0.6	Sugar	160	159	120	150	163	163	108	103	0.3	0.5
				L. acid	63			108				106		

\* Stock fed and fasted overnight, except in No. 65, which was maize fed and not fasted.

† Vagus nerves cut.

‡ (a) indicates atropine. (b) indicates ergotamine.

fasted for variable periods were mainly used, so that the percentages of glycogen in the liver varied from animal to animal. There are three experiments (Nos. 58, 61 and 63) in which the animals received 0.5 mg. ergotamine at the time of decerebration in region III, and in all of them the blood sugar level rose not at all or much less steeply than usual. In two experiments (Nos. 64 and 65) larger doses of ergotamine (0.8–0.9 mg.) still more markedly suppressed the hyperglycæmia. The behaviour of the blood lactic acid is of some interest in these experiments, for in three out of the five its percentage after four hours had scarcely risen above the initial value. With regard to the behaviour of the liver glycogen, it will be observed in four of the experiments that the percentage had scarcely changed after four hours. This is a very different result from that obtained following decerebration when no drugs are given and is probably of some significance. The table also contains a control experiment in which decerebration was performed in region II; the blood sugar percentage remained constant, the blood lactic acid percentage rose sharply and the liver glycogen percentage fell.

The results taken as a whole leave no doubt that ergotamine alone can completely counteract the effects of pontine decerebration in fasted stock-fed rabbits. This result is of interest in view of that obtained by Nitzescu and Munteanu [1932], who found that ergotamine prevents the development of hyperglycæmia following adrenaline.

The table also shows the results of three experiments (Nos. 51, 54 and 56) in which ergotamine (0.5–0.6 mg.) was injected along with atropine (10 mg.) into rabbits that had fasted overnight after being stock fed. In two of these animals the vagus nerves were also cut. The results with regard to the blood sugar level and the liver glycogen percentages are the same as after ergotamine alone, but they differ with regard to the blood lactic acid, which rose sharply. Apparently atropine annuls the effect which ergotamine has in preventing increase in the blood lactic acid percentage, but we do not wish to put much emphasis on this particular observation, since it is quite evident that, in rabbits at least, the blood lactic acid may vary considerably from unknown causes.

(3) *Behaviour of glycogen at intervals following pontine decerebration.* (Table III.)

In four experiments on fed animals (Nos. 31, 33, 34 and 41) decerebrated in region III, the blood sugar level rose in the usual manner, so that decided hyperglycæmia was present by the second hour. Meanwhile, the

TABLE III. The behaviour from hour to hour following decerebration of the percentages of sugar in the blood and glycogen in the liver and muscles.

Exp. No.	Wt. of animal (kg.)	Condition of animal	Level of decerebration	Blood sugar percentage										Glycogen percentage	
				Before decerebration	Hours after decerebration					Time of taking sample	Liver	Muscle			
					$\frac{1}{2}$	1	1½	2	2½				3		
31	1.3	High carbohydrate	III	215	180	190	235	275	375	395	430	Initial	4.9	0.5	
											1st hr.	5.2	—		
											2nd hr.	4.1	—		
											4th hr.	2.1	0.4		
32	1.8	Same	II	110	135	120	100	110	140	210	295	Initial	8.9	0.8	
											2nd hr.	7.3	—		
											3rd hr.	5.6	—		
											4th hr.	6.2	0.7		
33	1.7	Same	III	135	170	170	180	200	285	355	350	Initial	2.2	0.4	
											2nd hr.	2.6	—		
											3rd hr.	1.3	—		
											4th hr.	0.3	0.2		
34	2.2	Same	III	180	215	275	350	365	465	490	560	Initial	12.7	0.7	
											2nd hr.	11.7	—		
											3rd hr.	5.9	—		
											4th hr.	5.5	0.4		
41	1.5	Same (renal vessels ligated)	III	175	250	255	345	375	390	400	350	Initial	4.4	0.4	
											1st hr.	2.2	—		
											2nd hr.	1.8	—		
											3rd hr.	1.0	—		
											4th hr.	0.3	0.1		
35	2.3	Same (renal vessels ligated), atropine 10 mg. injected at (a)	III	200 (a)	340	325	435	460	490	510	470	Initial	11.9	1.0	
											1st hr.	6.0	—		
											2nd hr.	2.8	—		
											4th hr.	2.7	0.8		
37	1.5	Fasted atropine at (a)	III	160 (a)	280	255	195	170	220	245	350	Initial	0.5	0.8	
											1st hr.	0.3	—		
											2nd hr.	0.3	—		
											3rd hr.	0.2	—		
											4th hr.	0.0	0.5		

liver glycogen percentage remained practically unchanged in three out of the four experiments. After the second hour the rise in blood sugar percentage became very marked and the liver glycogen percentage rapidly decreased. There is evidence to show that the rapid increase in blood sugar in the later stages of the experiments was partly due to the handling of the liver entailed in removing pieces for analysis; thus, a similar rise occurred in an animal decerebrated in region II (No. 32). Administration of atropine at the time of decerebration (No. 35) did not prevent the development of hyperglycæmia when the glycogen percentage was high, but delayed it when this was low (No. 37).

The behaviour of the glycogen in the liver in these experiments confirms the conclusion previously drawn by Macleod and Donhoffer that the course and degree of decerebration hyperglycæmia is little dependent upon the rate at which it is discharged as glucose into the hepatic veins. No doubt, other things being equal, the percentage of blood sugar will tend to rise somewhat higher and to remain high for longer in glycogen-rich than in glycogen-poor animals, but this difference is by no means sufficiently pronounced to indicate that increased hepatic glycogenolysis is the function primarily responsible for the hyperglycæmia. For example, in Exp. 32 of the present series, in which decerebration was performed in region II, the blood sugar level did not change until after  $2\frac{1}{2}$  hours—when handling of the liver began to affect it—yet the fall in the liver glycogen percentage, up to 2 hours at least, was as great as in any of the other experiments.

As was to be expected, the percentage of glycogen in the leg muscles fell in all the experiments, and although this may have occurred in some more rapidly than in others, no relationship can be detected between the rate of this decline and that of the liver glycogen or between it and the rate at which the blood sugar percentage increased.

#### CONCLUSIONS.

1. When the percentage of liver glycogen is high in rabbits, atropine has only a slight retarding effect on the development of decerebration hyperglycæmia.
2. Under the same conditions, and also when the glycogen is low, ergotamine almost entirely prevents the hyperglycæmia.
3. In the majority of the experiments with ergotamine no increase occurred in the percentage of blood lactic acid following decerebration and the liver glycogen remained unchanged.

4. No parallelism could be demonstrated between the rate at which glycogen disappears from the liver following decerebration and the increase in blood sugar percentage.

The author wishes to thank Prof. J. J. R. Macleod for his advice and assistance during this investigation, which was undertaken at his suggestion.

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THE EFFECT OF DIET, OF INSULIN AND OF  
THYROXINE UPON THE ADRENALINE CONTENT  
OF THE SUPRARENAL GLANDS.

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THE EFFECT OF DIET.

In a recent communication Rosenblueth and Gayet [1932] report that rats placed on meat, fat or carbohydrate diet show no difference in their adrenaline content, and they conclude that the adrenaline store of the suprarenal glands is unaffected by the character of the diet. Being unaware of the work of these authors, I have been conducting similar experiments with, however, a somewhat different result.

In order to obviate any possible effect which climatic conditions might have on the results of the experiments, the investigation was carried out on three groups of animals, one during the hot Egyptian summer (all males), the second during the autumn (females and males), and the third during the beginning of winter (all females). The results obtained with these three groups showed no difference, and they will therefore be described together. The experiments were performed on 105 white rats of approximately the same weight and age; they were not fully grown, as it was hoped that the effect of diet would be more conspicuous in the growing animal. Before placing the animals on the different diets, in order to equalize their condition, they were kept for 7 days on a mixed diet composed of 68 p.c. dried bread, 20 p.c. meat containing fat, 10 p.c. milk and 2 p.c. salt mixture, all mixed into a paste with the addition of water.

On the 8th day the rats were separated into five batches of twenty-one rats each. The first batch continued to receive a mixed diet; the second was fed on lean minced meat which was slightly cooked for about 40 min. in thin layers in a hot-air oven; the third batch received mutton fat, which was separated from cellular elements by being melted on a water bath; the fourth batch was fed on raw rice, and the fifth was starved. All

the rats received a plentiful supply of water, and all except the fifth batch were given 2 g. of salt mixture and 5 c.c. of cod-liver oil to every 100 g. of the diet.

One third of the animals in each group was killed on the 9th–10th day of receiving the special diet, another third was killed on the 15th–16th day, and the remainder on the 20th–22nd day. Of the animals which were starved, some were killed on the 9th day, and all the rest on the 15th day. The animals were killed in an atmosphere of chloroform and coal gas [Crowden, 1929]. They died within 1–2 min. The suprarenal glands were at once excised, freed from connective tissue and weighed, and the adrenaline content of both glands together was estimated. The usual method of the chemical estimation of adrenaline by hydrochloric acid extraction, recommended by Folin, Cannon and Denis [1912–13], has been criticized by Baker and Marrian [1927], who found that extraction with trichloroacetic acid gave more reliable results. Wiltshire [1931] found that the addition of a trace of alanine retards the oxidation of adrenaline in solution, a result which I can entirely confirm. In view of this, the adrenaline was estimated as follows:

Both suprarenal glands were rapidly and finely ground with silver sand, with the addition of a few drops of 10 p.c. trichloroacetic acid containing 0.25 p.c. of alanine. About 1.5 c.c. of the trichloroacetic-alanine solution were added after the grinding and the whole mixture was agitated, the sand and precipitated proteins were allowed to settle, and the supernatant liquid was filtered off through a little wad of glass wool. The residue was extracted once more with 1.5 c.c. of the solution, filtered off, and then washed twice with a few c.c. of distilled water and a few drops of the trichloroacetic-alanine solution. The filtrates were centrifuged, and the small residue was once more thoroughly washed and centrifuged. Finally the whole extract was diluted to 25 c.c. in a measuring flask. The estimation of the adrenaline in the extract was carried out colorimetrically in a Duboscq colorimeter with the addition of Folin's "uric acid reagent," the standard being a 0.04 mg./100 g. uric acid solution.

The results of the experiments are represented graphically in Fig. 1 which shows that, in spite of the fact that there are considerable variations in the adrenaline content of the individual rats in each batch, there is not much difference between the control rats and the rats kept on protein diet, while the adrenaline content of the rats kept on the carbohydrate diet and on the fat diet is considerably diminished. The starved rats also showed diminution of the adrenaline content, confirming the results of all

previous observers [Vincent and Hollenberg, 1920; Mouriquand and Leulier, 1928; Rosenblueth and Gayet, 1932].

The comparison of the weight of the suprarenal glands of rats kept on different diets and the relative amounts of adrenaline per g. of gland

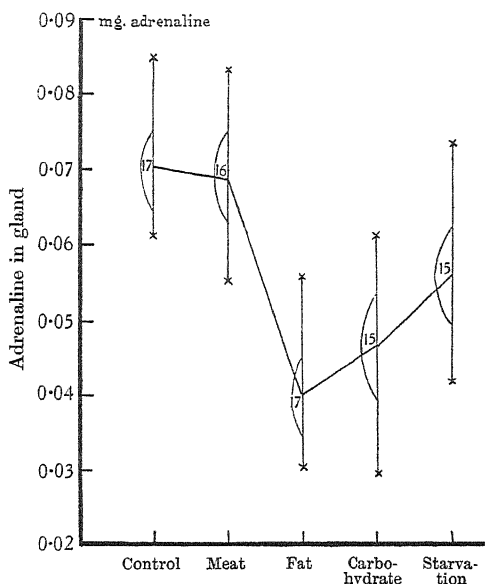


Fig. 1. The influence of different diets on the adrenaline content of the suprarenal glands. Vertical lines show the extent of the individual variations in the corresponding batches of rats. The averages are joined together. The figures in the segments represent the number of rats in each batch whose adrenaline content varies between the limits enclosed by the segment. There were twenty-one rats in each batch.

TABLE I.

	Weight of rats before diet g.	Weight of rats after diet g.	Weight of glands g.	Adrenaline content mg.	mg. adrenaline per g. gland	g. gland per kg. body weight
Control	2982	3276	0.745	1.459	1.958	0.227
Meat	2900	2976	0.708	1.373	1.939	0.237
Fat	2910	2289	0.519	0.827	1.595	0.227
Carbohydrate	3079	2512	0.393	0.991	2.525	0.156
Starvation	2940	1785	0.527	1.180	2.239	0.295

show additional points of interest. Table I gives the figures for the sum of all the rats in each group. On comparing the maxima and minima from the different groups, the result was found to be the same. The most striking difference is observed in the relative amount of adrenaline per g.

of gland in the case of fat, carbohydrate and inanition. In all three batches the weight of the suprarenal glands (col. 3) was found to be diminished as compared with the controls and the protein fed animals. With the fat diet, however, the diminution of the weight of the suprarenal glands was less pronounced than that of their adrenaline content, while during starvation and still more with the carbohydrate diet the weight of the glands diminished relatively more than the absolute amount of

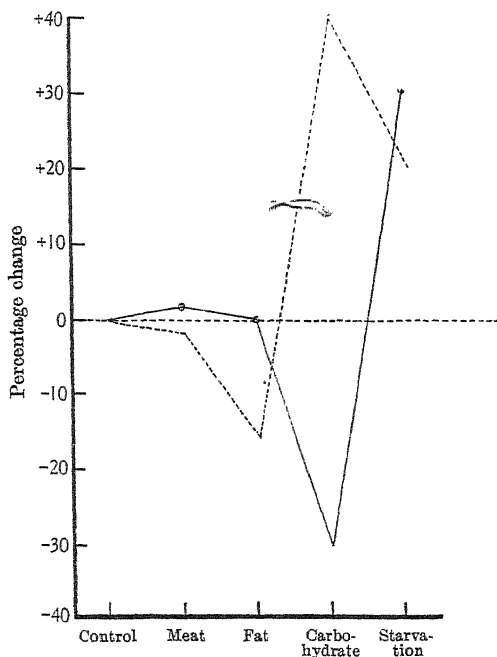


Fig. 2. The influence of different diets on the weight of the suprarenal glands in g. per kg. body weight (continuous line) and of their adrenaline store in mg. per g. of gland (dotted line). The calculation was made in comparison with the control animals.

adrenaline. Thus, with the fat diet, the adrenaline content per g. of gland diminished as compared with controls, while during starvation and even more with the carbohydrate diet it increased. These figures are of further interest when compared with the relative change of weight of the suprarenal glands per kg. body weight. In this respect there is no difference between the control batch and the protein fed rats, but in starvation the glands are relatively larger, and in the carbohydrate fed animals relatively smaller than in the controls. In the case of fat diet the weight of

the glands per kg. body weight does not differ from that of the normal. It is thus obvious that in the case of the fat diet the weight of the gland and that of the body diminished at the same rate, while in the case of the carbohydrate diet the glands diminished far more rapidly than the weight of the body. In starvation the weight of the body diminished more rapidly than the weight of the suprarenals.

Thus with the carbohydrate diet the suprarenals are markedly diminished in size but relatively heavily charged with adrenaline, while with the fat diet they are relatively poor in adrenaline. Fig. 2 represents the average results of all the experiments. On comparing individual animals it was found that the relative change in the adrenaline store of the suprarenal glands was not dependent upon the change in body weight, since rats which lost weight to a negligible extent showed as great a diminution in the adrenaline content as those which lost considerably more weight.

It is impossible to explain the difference between the results of these experiments and those of Rosenblueth and Gayet [1932], especially as these authors give no details of their experiments. It would be of interest to know the conditions under which their rats were kept, since the changes in weight of their animals are strikingly different from those of the present experiments. For instance, after 8 days of starvation the weight of their rats diminished by only 5.5 p.c., while in the present experiments, during the same period of starvation, the diminution was never less than 30 p.c. In the case of fat diet all the rats in the present experiments showed a diminution in weight, while the rats of Rosenblueth and Gayet either did not change in weight or, in the case of 35 days of feeding on fat, increased in weight.

#### THE EFFECT OF INSULIN.

There are various data in the literature concerning the activity of the suprarenal bodies under the influence of insulin, but no determinations were made of the adrenaline content. Most of the observers [Sundberg, 1923; Houssay and Lewis, 1923; Lewis and Magenta, 1925; Artundo, 1925; Ricci and Hilami, 1925; Houssay, 1925; Tada, 1931] consider that extirpation of both suprarenal glands leads to an increased sensitivity of the animal to insulin. Some authors [Cannon, McIver and Bliss, 1924; La Barre and Houssa, 1932] report that insulin hypoglycæmia leads to an increased secretion of adrenaline, the effect of which is to restore the blood sugar level by the mobilization of glycogen. Stewart and Rogoff [1923 *a*, *b*] hold the view that the adrenaline production is

unaffected by insulin. Thatcher [1926] investigated the effect of prolonged daily administration of insulin on the weight of the suprarenal bodies, and found it almost unchanged; the adrenaline content, however, was not determined.

In order to determine the effect of insulin on the load of adrenaline in the glands, two sets of adult rats, ten in each, were treated as follows: into one set over a period of 10–15 days small, definitely subconvulsive, doses (2 units at each injection) of insulin were injected, into the other, convulsive doses (10 units). Burroughs Wellcome's or Schering's insulin was used. In every case a control batch of the same number of animals of exactly the same weight was injected with an equal volume of a physiological saline solution. The results of these experiments are summarized in Table II.

TABLE II.

	Weight of rats after experiment g.	Weight of glands g.	Adrenaline content mg.	mg. adrenaline per g. gland	g. gland per kg. body weight
Subconvulsive doses of insulin	1809	0.442	0.768	1.741	0.244
Control	1788	0.319	0.654	2.048	0.177
Convulsive doses of insulin	2050	0.423	0.323	0.772	0.206
Control	2050	0.434	0.868	2.000	0.212
Thyroxine	1856	0.512	0.848	1.656	0.276
Control	1883	0.327	0.661	2.021	0.174

In every case the rats treated with subconvulsive doses of insulin showed a larger adrenaline content than their controls. Since the weight of the suprarenal glands was increased to a greater extent than the amount of adrenaline, the adrenaline content per g. of gland was diminished. The difference in the adrenaline content in the suprarenals was not large, but was definite enough to exclude the possibility of being due to individual variations, especially as this increase in adrenaline, as well as the increase in weight, was found in every single experiment. It is tempting to suggest, in view of the researches of the above-mentioned investigators, that the increase in weight of the suprarenal bodies and of their adrenaline content following the administration of insulin is due to a state of hyperactivity.

In the second set of animals, in which convulsive doses of insulin were used, the suprarenal bodies were found to have lost a considerable amount of their adrenaline store, as shown in Table II. The weight of the suprarenal bodies remained the same. It is impossible to say whether the depletion of the suprarenal bodies was the result of hypoglycæmia or of the prolonged convulsions of the animals. In order to determine the

mechanism of the action of the convulsive doses of insulin on the suprarenal glands, the effect of insulin was compared before and after section of the splanchnic nerves. Elliott [1912] demonstrated in cats that the right and left suprarenal bodies contained almost the same amount of adrenaline. The present work shows that this also holds for white rats. Denervation of the suprarenals on one side was carried out, under A.C.E. anaesthesia, through an abdominal incision, the wound being carefully closed after operation. The animals recovered a few minutes after the operation, and in about 30 min. they showed no difference from normal rats. Insulin was injected 2-5 hours after the operation. The animals were killed 2-4 hours after injection, and the adrenaline content of each suprarenal was determined separately. The average amount of adrenaline found in the ten normal glands was only 54.5 per cent. of that found in the denervated glands. In control animals, into which after the same operation physiological saline solution was injected, the innervated suprarenal contained on the average 71.5 p.c. of the amount found in the denervated gland. This result confirms that of Elliott [1912] and shows that the operative procedures had a marked effect on the innervated glands. The innervated suprarenals of the animals which received insulin were, however, depleted to a much greater extent than those of the controls which received only saline.

In order to make the effect of the insulin on the suprarenals independent of the effect of the operation, the experiments were repeated with the following modification. In each animal the left suprarenal was denervated while the right one was removed. Some of the rats operated upon in this manner received convulsive doses of insulin while others were kept as controls and received physiological saline instead. Two to four hours after the injection the animals were killed. In the case of the control animals the adrenaline content of the two glands was, in all cases, almost equal. On the other hand the adrenaline content of the denervated glands of the rats which received insulin was considerably diminished, the denervated gland containing on the average 70 p.c. of that found in the gland which was extirpated before the injection. This diminution must be attributed to an effect which insulin or the hypoglycaemia exerts directly on the suprarenal bodies. Besides this direct peripheral influence, however, there is also an influence through the nervous system.

On comparing the effect of insulin upon the normally innervated suprarenals as given in Table II with the effect upon the denervated glands, we find that in the first case the glands discharged on an average

63 p.c. of their adrenaline content, while the denervated glands discharged only about 30 p.c. These results, which were invariably obtained in a large number of experiments, show that insulin exerts a double influence. It stimulates the suprarenals through the central nervous system along the splanchnic nerves as well as peripherally by acting upon the gland itself. This conclusion is supported by experiments in which both glands were left in the animals, but one was denervated while the other was left intact. In control animals the innervated gland contained about 70 p.c. of adrenaline as compared with the denervated side, while in the animals which received insulin it contained 50 p.c., showing that under the action of insulin it lost more adrenaline than from the operation alone. In these experiments both the innervated as well as the denervated suprarenals were left *in situ*, so that the difference in their adrenaline content was due to the central impulses reaching one gland and not the other.

#### THE EFFECT OF THYROXINE.

In view of the fact that the effect of thyroxine is in many respects similar to that of adrenaline, it was of interest to find whether repeated administration of thyroxine would lead to any change in the adrenaline content of the suprarenal glands. For this purpose ten normal rats were each given injections of 0.5 mg. of thyroxine for 10-15 days. Similarly ten other rats of exactly the same weight had injections of physiological saline solution. The animals were killed and the suprarenals were treated in the same way as before. The results of these experiments are given in Table II. In every case the animals that had thyroxine had heavier suprarenal glands which contained more adrenaline. But the increase in weight of the glands was proportionately greater than that of their adrenaline so that, when calculated per g. of gland, the adrenaline content was relatively diminished. Thus the effect of thyroxine on the suprarenals and their adrenaline store is similar to the effect of small subconvulsive doses of insulin.

#### CONCLUSIONS.

1. The adrenaline content and the weight of the suprarenal glands are affected by variations in the diet. While high protein diet has no marked effect, fat diet leads to a diminution of the absolute content of adrenaline and of the total weight of the glands. The relative amount of adrenaline per g. of gland is also slightly diminished. Carbohydrate diet also leads to a diminution of the absolute content of adrenaline; the weight of the suprarenals is strikingly diminished so that the relative amount of adrenaline per g. of gland is considerably increased.



2. Repeated administration of insulin in subconvulsive doses leads to an increase in the weight of the suprarenals and of their adrenaline content. In convulsive doses, insulin leads to a depletion of the adrenaline store. This effect of insulin is produced through the nervous system as well as by peripheral action on the suprarenal bodies.

3. Repeated injection of thyroxine also leads to an increase in weight and of adrenaline content of the suprarenal bodies.

This research was performed at the suggestion of Prof. G. V. Anrep. I am greatly indebted to him and to Dr A. Hassan for their helpful criticism and advice.

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THE CORRELATION BETWEEN THE ACTION OF  
INSULIN AND ADRENALINE UPON THE  
MUSCLE AND LIVER GLYCOGEN.

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It was shown by Dudley and Marrian [1923] that the hypoglycæmia which follows an injection of insulin into a normal animal is accompanied by a mobilization of glycogen from the liver as well as from the muscles. The hyperglycæmia caused by injection of adrenaline is also associated with a depletion of the glycogen stores. There is, however, no agreement as to whether in the latter case it is the liver or the muscle glycogen which is chiefly affected. Ohara [1925] found that adrenaline brings about a hyperglycæmia in rabbits even after their glycogen store has been greatly reduced by starvation. Hyperglycæmia has also been produced in hepatectomized dogs, and in this case it was found that the glycogen store of the muscles was considerably diminished. Ohara also found that in normal dogs a single injection of adrenaline reduced the glycogen content of both the liver and the muscles. On the other hand, Cori and Cori [1928] state that, in fasting rats, adrenaline leads to an increase in the liver glycogen and a decrease in the muscle glycogen, an observation which was confirmed by Blatherwick and Sahyun [1929]. Goldblatt [1929] finds that adrenaline in starving young rabbits diminishes only the glycogen store of the muscles, leaving the liver glycogen unaffected, while Eadie [1929] claims to have shown that adrenaline has no effect on the muscle glycogen although it reduces the liver glycogen to vanishing point.

Previous experiments by one of us [Gohar, 1933] on rats have demonstrated that an injection of a convulsive dose of insulin leads to a considerable diminution of the adrenaline content of the suprarenal glands. Experiments by other workers (for literature see Gohar [1933])

suggest that an increased secretion of adrenaline is evoked by insulin. Many authors have stated that suprarenalectomy leads to an increased sensitivity of the animal to insulin [Sundberg, 1923; Houssay and Lewis, 1923; Lewis and Magenta, 1925; Artundo, 1925; Ricci and Hilami, 1925; Houssay, 1925; Tada, 1931]. These observations suggest that the mobilization of glycogen under the action of insulin may be assisted to an unknown degree by a concurrent increase in the secretion of adrenaline. The experiments described in the present communication were performed in order to determine the part which the suprarenal glands may possibly play in the action of insulin, especially with reference to the depletion of the stores of glycogen.

#### METHOD AND EXPERIMENTAL PROCEDURE.

A comparison was made between the effect of insulin on the liver and muscle glycogen in suprarenalectomized rats and rats on which a sham operation had been performed. It was shown by Karczag, Macleod and Orr [1925] that white rats, which are kept under strictly identical conditions of feeding and caging, are suitable animals for the study of the influence of various factors upon the glycogen store and upon the distribution of glycogen between the different organs. Since the figures given by these authors seem to indicate that the percentage of liver and muscle glycogen is less susceptible to variations in females than in males, we performed our experiments exclusively on female white rats. The age difference between the animals did not exceed 7 days, and all the rats were taken from a stock which had been bred in the laboratory for many generations. As recommended by Macleod and his co-workers, the animals were starved for 40 hours before the experiments, only water being allowed. They were then kept for not less than a fortnight on a mixed diet of the following composition: 60 p.c. bread, 20 p.c. meat, 15 p.c. milk, 3 p.c. cod-liver oil, 2 p.c. salts. The meat contained some fat and was minced, slightly roasted and kept in a refrigerator until required. In order to ensure a regular and simultaneous feeding, the animals were fed three times a day, at 7 a.m., 1 p.m. and 7 p.m. The food was left in the cages for 1 hour on each occasion. On the days of the experiments the animals were deprived of all food. In confirmation of the observations of Karczag, Macleod and Orr, there were no great variations in the muscle and liver glycogen in these animals, provided they were killed the same interval of time after their last meal. This was especially the case with the muscle glycogen.

On the days of an experiment two, or in some cases four, rats were selected of exactly the same weight and left without food. On one of the pair a double suprarenalectomy was performed through lumbar incisions under c. + e. anæsthesia. A sham operation was performed on the other rat, both suprarenals being exposed but left intact. Care was taken to keep both animals under anæsthesia for the same length of time. The wound was carefully closed after the operation, which usually lasted for about 10 min. The animals invariably recovered completely from the anæsthesia within 5 min. Although every pair of rats was always treated in exactly the same manner, the operations and the estimations of glycogen in the different pairs were not always performed after the same period of fasting, and therefore the amount of glycogen, although strictly comparable in the rats belonging to a single pair, showed considerable variations in animals which had the operation on different days. This difference may also have been due in part to the considerable changes in the daily temperature and the climatic conditions. White rats are particularly suitable for the purpose of these experiments since Vincent [1924] could not demonstrate in them the presence of any accessory chromophile bodies.

The actual experiments were performed in the following manner. Two hours after the operation equal convulsive doses (4–8 units) of insulin were injected into both rats. Usually one injection was sufficient. In a few cases this was followed by a second injection, about 1 hour after the first. When one of the pair, invariably the suprarenalectomized, became comatose it was killed with a blow on the head. The other rat was allowed to survive for exactly the same length of time after the injection of the insulin as the first rat. Immediately after killing an animal its liver and thigh muscles were rapidly removed and prepared for the estimation of glycogen. Great care was taken to keep the details of the preparation and analysis constant for all the specimens. Completely untreated rats were occasionally used as controls; they were kept under the same conditions, left to starve for the same time, and in some cases kept under anæsthesia for as long as the operated rats. This provided us with information as to the percentage of glycogen which is likely to be left in the liver and muscles before the operation.

The organs used for the estimation were rapidly weighed; 1 c.c. of 60 p.c. KOH was added for each g. of tissue, and the mixture was placed in a vigorously boiling water bath under reflux for 2 hours. After being cooled, the liquid was diluted with 2 volumes of water, treated with 4 volumes of 96 p.c. alcohol and well stirred. The precipitate was allowed

to settle and then collected on a filter paper, washed once with a mixture of 1 volume of 15 p.c. KOH with 2 volumes of 96 p.c. alcohol, and afterwards washed twice with 66 p.c. alcohol. The precipitate was boiled in water, allowed to cool, neutralized with dilute HCl and filtered. After the filter paper had been washed several times with small amounts of water, the filtrate was made to a definite volume, and the glycogen concentration in the solution was determined. In preliminary experiments the glycogen determinations were carried out according to the usual method, *i.e.* hydrolysis in 2.2 p.c. HCl for 3 hours in a boiling water bath, followed by determination of the sugar by one of the recognized methods. The Hagedorn and Jensen method was used in most cases. It was soon found, however, that when the glycogen concentration is very small this procedure fails to give satisfactory results. In our experiments the amount of glycogen in animals treated with insulin or adrenaline was invariably very small. In the amounts of tissue used for analysis it was frequently only a few mg., and the solution used for the determination could not be conveniently concentrated so as to give satisfactory results with sugar-reducing methods.

Several investigators [Nerking, 1901; Kerley, 1930] have pointed out that the hydrolysis of glycogen does not give a quantitative yield of glucose. We were able to confirm this observation on a sample of pure glycogen in concentrations of the order obtained in our preliminary experiments on the liver and muscles of insulin-treated rats. The percentage loss of glucose during hydrolysis varied with the strength of the acid, the length of hydrolysis, and even with the concentration of glycogen, becoming less as the concentration increased. This introduced a factor which could obviously not be controlled. We therefore decided to use the polarimetric method of glycogen determination, which has been claimed to be more exact than sugar-reducing methods for low concentrations of glycogen.

Harden and Young [1902] have shown that, in low concentrations of glycogen where the opalescence does not interfere with the rotation, a fairly constant specific rotary power can be obtained, even for specimens of glycogen from different sources. Pflüger [1906] also recommended the direct polarimetric determination of glycogen. Using Hilger's polarimeter, and solutions of pure glycogen in concentrations similar to those obtained in our preliminary experiments, we obtained completely satisfactory results. The specific rotary power of pure animal glycogen was taken as  $[\alpha]_D^{20} = 200$  or  $[\alpha]_{Hg}^{20} = 236.2$ . The accuracy of this method diminishes with increase in the glycogen concentration, owing to opal-

escence. Below 0.4 p.c. the method was found to be accurate. Since the concentrations of glycogen in our experiments were below this figure, we adopted the polarimetric method for our work in preference to the sugar-reducing method.

#### EXPERIMENTAL RESULTS.

A comparison between the percentage of glycogen in the liver and muscles of suprarenalectomized and sham-operated rats, after the injection of convulsive doses of insulin, is given in Table I. These results and many others which were exactly similar show that in all cases the percentage of muscle glycogen in the suprarenalectomized rats injected with insulin is distinctly higher than in sham-operated rats which were treated

Weight of each rat of the pair g.	Percentage of glycogen			
	Suprarenalectomized		Sham-operated	
	Liver	Muscle	Liver	Muscle
195	0.00*	0.27	0.00*	0.17
220	"	0.32	"	0.20
270	"	0.07	"	0.03
240	"	0.08	"	0.05
240	"	0.15	"	0.06
225	"	0.17	"	0.12
225	"	0.28	"	0.17
200	"	0.27	"	0.16
200	"	0.09	"	0.04

\* Figures below 0.02 are given as zero.

in a similar manner. The liver, on the other hand, is almost completely depleted of glycogen in both cases. At first sight these results appear to show that, while the mobilization of the glycogen of the muscles is influenced by the suprarenal glands, the glycogenolysis in the liver is directly brought about through the influence of insulin and that it is independent of the discharge of adrenaline. Further observations, however, show that this is not the case.

The depletion of the liver glycogen is not due to starvation. This is shown by the figures in Table II, where a comparison is made between the percentage of liver glycogen in suprarenalectomized rats and sham-operated animals which were injected with insulin with that of livers of animals which were either simply starved or else starved and then anaesthetized for the same length of time as the operated animals. While the liver glycogen of the insulin-injected rats was reduced to vanishing point, the livers of the starved non-anaesthetized rats contained a considerable amount. The administration of the anaesthesia and the actual

operation could also not explain the almost complete disappearance of glycogen from the liver, as was shown by comparing the glycogen content of non-anæsthetized and non-operated animals with that of rats which were anæsthetized and operated upon but not injected with insulin. For this purpose a number of rats were subjected to A.C.E. anæsthesia for 10 min. each; while they were under the anæsthetic a sham suprarenal-ectomy was performed. The rats were then left for about 5 hours. Other rats of exactly the same weight were used as controls. The liver glycogen was found to be reduced by 40–50 p.c. in the anæsthetized rats. In no case was the depletion greater than this. These results make it evident that, although the anæsthesia and the operation led to a considerable diminution of the glycogen store of the liver, this could not explain the

TABLE II.

Weight of each of the four rats g.	Percentage of glycogen							
	Control rats		Suprarenalecto- mized rats		Sham-operated rats		Rats injected with adrenaline	
	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
195	0.73	0.29	0.00*	0.27	0.00*	0.17	0.00*	0.04
240	0.78	0.17	„	0.15	„	0.06	„	0.05
200	0.37	0.12	„	0.11	„	0.04	„	0.03
198	1.20	0.21	„	0.25	„	0.16	„	0.07
	Mean	0.197		0.195		0.107		0.047

\* Figures below 0.02 are given as zero.

almost complete depletion of it observed after the injection of insulin. As regards the muscle glycogen, it was found to be unaffected by the length of the anæsthesia employed and the operation performed.

It can be seen from Table II that it is not only the injection of convulsive doses of insulin which leads to a complete exhaustion of glycogen from the liver but that adrenaline produces the same effect. One of a pair of rats of exactly equal weight was injected with 0.05–0.08 mg. of adrenaline, a dose of the order experimentally found to be normally present in the two suprarenal glands of similarly kept rats; the other rat served as a control. The rats were killed simultaneously 2 or 3 hours after the injection, and their livers and muscles were analysed for glycogen. The degree of depletion is striking. There is thus no reason to consider that the adrenaline secretion evoked by insulin, or by the after-effects of its administration, is without influence upon the mobilization of the liver glycogen. However, since insulin leads to as complete a depletion of the liver glycogen store even in suprarenalectomized animals, it must be concluded that the glycogenolysis in the liver is determined on the one

hand by insulin, or by the symptoms following its administration, and on the other hand by the increased secretion of adrenaline which it causes.

In the case of the muscle glycogen the results differ to a considerable extent. Insulin seems to play no direct rôle in the mobilization of the glycogen. This is shown by comparison of the percentages of muscle glycogen in the first two parts of Table II, which show that in the supra-renalectomized animals injected with insulin the amount of glycogen left in the muscles at the end of the experiments is approximately the same as in the untreated controls. The mean percentage for the suprarenal-ectomized rats in the four experiments is given in Table II as 0.195 p.c., and for the untreated controls as 0.197 p.c. The figures given in the table were taken from four representative experiments. A great number of other experiments not reproduced in Table II gave exactly similar results. The four in the table were selected from those which showed the greatest difference in the absolute amount of glycogen present in the muscles and liver of the control animals. Four rats of exactly the same weight were used for each experiment. A comparison of the second and third part of Table II shows that while convulsive doses of insulin fail to cause a mobilization of glycogen from the muscles in the absence of the suprarenal glands, in sham-operated animals the mobilization of the muscle glycogen is considerable. In the four experiments given in Table II the average percentage of muscle glycogen in the suprarenal-ectomized rats was 0.195 p.c., and in the sham-operated ones 0.107, an average diminution of about 45 p.c. Other experiments showed the same order of diminution.

These results, together with those given in Table I, show that the mobilization of the muscle glycogen caused by insulin is not brought about directly by the insulin, but through the increase in the adrenaline discharge which it produces. Further evidence in favour of this conclusion is provided by the comparison of the time which elapses between the injection of insulin and the onset of convulsions in the suprarenal-ectomized rats and in the sham-operated rats. Without a single exception the convulsions invariably started in sham-operated rats from 45 to 90 min. later than in the others. In one case the insulin used happened to be a less potent preparation than usual and a big dose had to be injected. The suprarenal-ectomized animals had convulsions and died about 15 hours after the injection, but the sham-operated rats did not even have convulsions. The general condition of the suprarenal-ectomized rats always aggravated more rapidly, coma developed earlier, and they died sooner than the sham-operated rats. This observation, together with the fact that the glycogen content of the muscles of the suprarenal-ecto-



mized rats is always higher than that of the sham-operated animals, indicates an earlier lowering of the blood sugar level in the former, owing to the diminution of the available source of glycogen in the absence of the suprarenal gland.

It is evident that the convulsions were due to hypoglycæmia, and not to the operative procedure, because of their typical nature and also because injections of glucose caused a rapid relief. Those animals which were not killed and which did not die after the injection of insulin were found to be in good condition many weeks after the operation.

It might be thought that, since the suprarenalectomized and the sham-operated rats were always killed the same time after injection of insulin, and since the suprarenalectomized animals had convulsions for not less than one hour longer than the sham-operated rats, the former would have shown a greater diminution in the glycogen content of their muscles. However, this is not the case, indicating that the diminution of the muscle glycogen content could not possibly be explained by the difference in degree of muscular activity. The muscular activity seemed to be in any case insufficient to produce an appreciable change in the glycogen store of the muscle, as shown by a comparison of the first and second parts of Table II.

#### CONCLUSIONS.

1. Injection of convulsive doses of insulin into rats, which are kept under strictly controlled conditions, is followed by an almost complete depletion of the liver glycogen and a considerable diminution of the muscle glycogen.

2. In suprarenalectomized animals, insulin leads to a similar diminution in the liver glycogen but apparently has no effect on the muscle glycogen.

3. Injection of adrenaline depletes the liver of its glycogen and considerably diminishes the glycogen in the muscles.

4. It is suggested that the depletion of the liver glycogen is determined by the synergetic action of insulin and adrenaline, either of them alone giving the same result as both together, while the depletion of the muscle glycogen is brought about by the insulin only indirectly through an increased activity of the suprarenal glands.

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THE EFFECT OF SPLANCHNOTOMY AND OF  
PHLORRHIZIN ON DECEREBRATION  
HYPERGLYCÆMIA<sup>1</sup>.

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IN discussing the nature of the nervous disturbance which is responsible for the development of hyperglycæmia following pontine decerebration in rabbits, Donhoffer and Macleod [1932] did not have available the results of experiments performed on animals in which the splanchnic nerves had been cut some days previous to decerebration. The present paper contains such results. It also records those obtained when the rabbits received large doses of phlorrhizin prior and subsequent to decerebration. This type of experiment was undertaken for the following reason. It was shown by Donhoffer and Macleod that hyperglycæmia which followed pontine decerebration in previously fasted rabbits was comparable in degree with that observed by Cori and Cori [1928] to be produced when 2 g. of glucose per kg. an hour were given by continuous intravenous injection into amytalized rabbits. Since this amount exceeds by many times that which could be accounted for by the glycogen which disappears from liver and muscles following pontine decerebration, it was suggested that the process of gluconeogenesis in the liver had become excessively active. The only alternative explanation for the accumulation of extra sugar in the blood is that the utilization of this substance in the tissues has become lessened. It was found, however, that the oxygen consumption, as well as the respiratory quotient, was not demonstrably influenced by the decerebration, indicating that the oxidation of carbohydrate had not become depressed. It may be noted here in passing that the stability of the respiratory quotient would also seem to indicate that gluconeogenesis had not become increased. If the oxidation of carbo-

<sup>1</sup> This investigation was carried out at the suggestion of Prof. J. J. R. Macleod.

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hydrate is not depressed, a lower rate of usage of glucose in the tissues might depend on a raising of the threshold at which glucose passes into them from the blood. A disturbance of this nature might also be responsible for the hyperglycæmia caused by adrenaline injections into fasting animals.

Even if the rate of sugar production by the liver were undisturbed, a small raising of the threshold of migration of glucose from blood to tissues could cause a very decided increase in the blood sugar level. It is believed that the primary cause of the glycosuria which follows phlorrhizin injections is a lowering of the sugar threshold in the kidneys, and there is not wanting some evidence that this drug also causes sugar to appear in such secretions as the bile, pancreatic juice, etc. [R. G. Pearce, 1916]. If this be the case then phlorrhizin may also lower the threshold for sugar in the muscles, and it seemed of interest to see what effect it would have on decerebration hyperglycæmia. Since, however, the lowering of the renal threshold is the most outstanding effect of the drug, as judged by comparison of the percentages of glucose in the arterial and venous blood [Nash, 1922] of the kidneys, it was necessary in certain of the present experiments to remove the kidneys by ligation of the renal vessels. The experimental procedure and the chemical methods employed in this investigation have been essentially the same as those used by Donhoffer and Macleod.

## RESULTS.

### (1) *The effect of splanchnotomy.*

Splanchnotomy was performed 1-8 days prior to the decerebration experiment, the animals being anæsthetized by amytal. They were then put on a normal mixed diet, the last meal being given about 18 hours before the decerebration experiment, except in Exps. 35 and 36, when they were only fasted overnight.

A total of eight experiments were carried through, three of them on animals decerebrated through the middle of region III, three through the posterior part of the same region, one through region IV, and one between regions II and III. The results are given in Table I. In two out of the pontine decerebration experiments (viz. Nos. 53 and 54) the blood sugar failed to rise significantly, the last blood sugar value of Exp. 54 being high because it was taken from the heart when the rabbit was in a dying condition. In the third experiment of this group (viz. No. 36) the blood sugar rose from 120 mg. per 100 c.c. blood to 215 within three hours, which is much less than that usually observed.

TABLE I. The effect of pontine decerebration on splanchnotomized rabbits.

Exp. No.	Region of decerebration	Blood sugar at hourly intervals in mg. per 100 c.c. blood							Glycogen p.c. in			
		Normal							Liver		Muscle	
			1	2	3	4	5	6	Before	After	Before	After
F 36	3	120	135	180	215	215	205	—	0.74	0.33	0.19	0.22
53	3	115	120	125	130	125	120	145	1.22	0.73	0.61	0.52
					H							
54	3	120	125	140	155	250	—	—	1.45	0.47	—	0.59
21	Post. 3	85	160	160	150	—	—	—	0.91	nil	0.34	0.31
F 35	Post. 3	150	175	150	150	100	—	—	0.84	0.01	0.25	0.28
	Slightly post. 3											
55	3	125	130	135	130	120	140	125	3.27	1.60	0.76	0.16
31	Ant. 3	120	120	120	120	130	105	—	—	—	—	—
20	4	115	132	172	—	215	—	—	2.43	0.49	0.52	0.51

F=rabbit fasted overnight.

H=blood sample withdrawn from heart.

In the three experiments (viz. Nos. 21, 35 and 55), where the lesion was made through the posterior part of the pons, it is probable, though not certain, that the diabetic centre was touched at some point along the line of decerebration. In only one of these three experiments (viz. No. 21) was there a small degree of hyperglycæmia, the blood sugar rising from 85 mg. per 100 c.c. blood to 160. In all these experiments save one (No. 55) the liver glycogen percentage fell as usual, and in all there was very little, if any, change in that of the muscles.

The results of the experiment (No. 31), in which the decerebration was through the anterior portion of the pons, are doubtful, since it occasionally happens that under these conditions the blood sugar level is not disturbed.

The results, as a whole, clearly show that splanchnotomy prevents the development of hyperglycæmia following pontine decerebration, and they indicate that the muscle glycogen is not diminished to the same extent as when the splanchnic nerves are intact.

## (2) The effect of phlorrhizin.

Rabbits which had been fed on an ordinary mixed diet were fasted overnight or for longer periods (18–36 hours). Twenty to thirty minutes prior to decerebration under amytal anaesthesia 0.75 g. phlorrhizin was given subcutaneously and, where indicated in the table (Table II), the

TABLE II. Effect of phlorrhizin on decerebration hyperglycemia.

TABLE II. Effect of parathion on acetylcholinesterase activity of p.p.v. venom.

Exp. No.	Region of decerebration	Initial dose of de-phlorrhizin g.	Blood sugar at hourly intervals in mg. per 100 c.c. blood										Liver glycogen p.c.		Glycosuria present	Remarks Kidney vessels not tied	
			Before							After	p.c.						
			Normal	1	2	3	4	5	6				7				
F 45	3	0.75	130	115	*05	95	135	*175	160	175	—	—	—	—	—	—	—
F 46	3	"	115	130	130	140	*140	155	215	—	H	5.00	1.25	"	"	"	"
38	3	"	60	150	210	250	—	210	190	—	—	—	1.90	—	—	Kidney vessels tied	"
F 48	3	"	130	270	270	295	310	*330	320	—	—	1.95	nil	—	—	"	"
F 49	3	"	115	185	170	*200	290	*—	360	—	—	3.94	nil	—	—	"	"
34	2	"	110	110	110	105	105	85	—	—	—	—	nil	—	—	"	"
44	2	"	120	190	165	*140	165	145	—	—	—	—	—	—	present	Kidney vessels not tied	"
37	Ant. 3	0.5	110	120	165	170	205	220	—	—	—	—	1.75	—	—	Kidney vessels tied	"
40	Post. 3	"	180	200	245	270	295	310	—	—	—	—	—	—	present	Kidney vessels not tied	"
47	4	0.75	175	260	*205	185	200	220	*215	—	11.43	6.97	"	"	"	"	"

\* Subsequent subcutaneous injections of 0.25 g. phlorrhizin.

F = Rabbit fasted overnight.

H = Blood sample withdrawn from heart.

kidney vessels were ligated. A sample of liver was also removed for estimation of glycogen by the Pflüger method, and a sample of blood withdrawn for estimation of the blood sugar. In Exps. 37 and 40 only 0.5 g. phlorrhizin was given.

Asterisks in the table mark subsequent doses of 0.25 g.

After decerebration blood samples were withdrawn from the ear at intervals of one hour. The experiment ended with the removal of a piece of liver for glycogen estimation and, when the kidney vessels were not tied, of a sample of urine to test for the presence of sugar by Benedict's method.

In five of the experiments the lesion was definitely through the centre of region III. In two of them (viz. Nos. 45 and 46) the kidneys were not tied off and no significant hyperglycæmia developed. In the remaining three experiments (Nos. 38, 48 and 49), where the kidneys were ligated, a definite and prolonged rise in blood sugar percentage followed decerebration.

Where decerebration was performed at other levels the results were not so orderly.

In Exp. 37 the line of decerebration was at the anterior border of the pons, so that it is impossible to say whether it was the phlorrhizin or the anterior position of the lesion which was responsible for there being only a moderate rise in the blood sugar level. In Exp. 40, in which the decerebration was through the posterior portion of the pons and the kidney vessels were intact, hyperglycæmia became developed, possibly because only a small amount of phlorrhizin was injected at the beginning of the experiment. The results of Exps. 34 and 44, in which decerebration was distinctly anterior to the pons, the blood sugar level did not change except temporarily, whether or not the renal vessels were tied, showing that this operation and the administration of phlorrhizin do not in themselves have an effect on the blood sugar level.

#### CONCLUSIONS.

1. Pontine decerebration in fasted rabbits in which the splanchnic nerves have been cut several days previously is not followed by an increase in the blood sugar percentage.

2. Injection of large doses of phlorrhizin does not prevent the development of decerebration hyperglycæmia when the renal vessels are ligated, but does so when these are intact. This result supports the view that phlorrhizin acts directly on the kidneys.

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THE INFLUENCE OF VAGAL STIMULATION UPON  
CONDUCTION THROUGH THE BRANCHES OF  
THE A.-V. BUNDLE IN THE DOG.

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It is a common observation that when the vagus nerves are stimulated an impulse passing from the auricle to ventricle travels with difficulty; it either suffers delay or is partially or completely blocked, according to the degree of stimulation employed. The disturbed impulses usually reach the ventricle in a perfectly normal manner, giving rise to normal ventricular complexes. On occasions, however, they travel abnormally, reaching one ventricle before the other, so that abnormal complexes occur during the stimulation. These take place both when the block between the auricle and ventricle is complete [Einthoven, 1906; Kahn, 1909; Kraus and Nicolai, 1910] and also when it is partial; but recorded examples of the latter are few. Hering [1909] reported aberrant ventricular complexes in a dog during vagal stimulation, the ventricular beat being preceded by an auricular beat. Kraus and Nicolai [1910] stimulated the right auricle during vagal inhibition and obtained aberrant ventricular complexes. Rothberger and Winterberg [1911] have published curves in which each aberrant ventricular complex was preceded by an auricular wave. In these experiments, however, the vagal effect was complicated by a simultaneous accelerator stimulation. Einthoven and Wieringa [1914] published curves from dogs during morphine poisoning. Certain of these during partial heart block show aberrant ventricular complexes to supraventricular stimuli. This rhythm was abolished by atropinization. In 1915 Wilson reported a clinical case in which a perfectly normal cardiac rhythm could be changed by deep breathing or vagal pressure into one in which the pacemaker was situated

<sup>1</sup> Working on behalf of the Medical Research Council.

high up in the A.-v. node and the ventricular complexes were aberrant. When this rhythm occurred spontaneously it could be abolished by atropine. In all these instances the impulse giving rise to the aberrant beat arose either in the auricle or high up in the A.-v. node. It travelled presumably through the main branch of the bundle and the bundle branches. The explanation which was offered by Einthoven and Wieringa [1914] for such beats was that the vagus nerves influence conduction not only in the A.-v. node and bundle, but also in the bundle branches, and that the group of fibres supplying one of the bundle branches reacted more strongly so that conduction in this branch was relatively more disturbed than in the other, and aberrant ventricular complexes thus arose from supraventricular stimuli.

As far as concerns those observations in which complete block is present, the idioventricular rhythm may arise high in the A.-v. bundle and the impulse be delayed in one of the bundle branches by vagal stimulation. It may equally well be that the idioventricular rhythmic centre is situated in one of the bundle branches. Both conditions would give rise to abnormal ventricular complexes.

These observations suggest that vagal stimulation influences branch conduction. Lewis [1914] has shown, however, that the chief action of the vagus is at the A.-v. node or its immediate neighbourhood. It seemed desirable therefore to find out whether or not vagal stimulation delays conduction in the bundle branches.

The method we have used depends upon the following considerations.

Lewis and Rothschild [1914] have shown that when the left ventricle of a dog is stimulated there are two paths open for the impulse to take as it travels to the right ventricle. It can pass entirely through muscle or it can reach the Purkinje tissue lining the left ventricular cavity, pass up the left and down the right bundle branch, and spread through the Purkinje tissue lining the right ventricle and so reach the muscle. Owing to the slow rate of conduction through muscle, compared to the high rate through Purkinje tissue, they found that the impulse must travel to the distant parts by way of the Purkinje system and the bundle branches, as it reaches these regions before it could do so if it travelled by muscle tracts only. We can therefore liberate an impulse which in its course will travel through the bundle branches, and by studying its rate of propagation both normally and during vagal stimulation, determine whether such stimulation has any influence.

Aberrant ventricular complexes are due to the delayed arrival of the excitation wave at the termination of one or other bundle branch.

Lewis [1916] showed that in the right bundle branch block the first part of the Q.R.S. complex is due to the spread through the left ventricle; in left bundle branch block, to the spread through the right ventricle. If the pure right ventricular effects and pure left ventricular effects were added algebraically in their proper time relations, a perfectly normal Q.R.S. complex was obtained. Wilson and Herman [1921] using the data of Lewis have determined the effect of shifting the relative position of the dextro- and lævocardiogram upon the composite wave, and have shown that a shift of 0.005 sec. produces curves which are transitional between normal and bundle branch block curves; they are aberrant in form. From these observations it appears evident that the delay in conduction through one bundle branch must be at least of the order of 0.005 sec. greater than that through the other if aberrant beats are to occur. Even if it is assumed that the disturbance in conduction produced by vagal stimulation is confined to one bundle branch, the delay, namely 0.005 sec., should be appreciated by the method we have employed. The more likely result of such stimulation, however, is a delay in both branches, with that in one branch greater than that in the other. In these conditions a considerable delay in the passage through both bundle branches would be expected, and would be clearly seen in the observations which we have made.

#### METHOD.

Dogs of about 10 kg. were used, and were anaesthetized with morphia followed by paraldehyde and, when necessary, a sufficiency of ether. Under artificial respiration the thorax was opened and the heart exposed. The pericardium was cut open and sutured to the chest wall, forming a cradle in which the heart freely beat. A radiant heater suitably placed maintained constant body temperature. The vagus nerves were exposed in the neck and stimulated by means of a faradizing current; they were usually cut.

The arrival of the excitation wave at different points on the ventricular surface was determined by placing an electrode upon the muscle and connecting it to a string galvanometer. The electrode consisted of an outer silver tube about 1/8 in. diameter, through the centre of which a fine insulated silver wire passed. The outer tube and inner wire formed the two leads to the galvanometer. The inner wire projected about 1 mm. beyond the outer tube and when in position upon the heart penetrated the muscle. This form of electrode was employed as it gives a very similar electrical response wherever it was placed upon the ventricular

muscle. When it was connected to the galvanometer so that activity beneath the inner wire caused an upward deflection, the record was diphasic, a small slowing rising upward deflection being followed abruptly by a large rapidly falling downward deflection. The downward deflection was therefore associated with activity beneath the outer silver tube. The beginning of activity beneath the small central wire was not to be readily appreciated so we have used the beginning of the large deflection for our measurements. With the record of this deflection, a lead II electrocardiogram was recorded simultaneously. This serves not only as an index of the constancy of the rhythm but allows the arrival of the excitation wave on the ventricular surface to be correlated with a "standard" position in lead II.

Lewis and Rothschild [1914] in their observations employed two separate electrodes, one upon the muscle, the other upon indifferent tissue nearby. We have compared the time measurements as obtained by this method with those by the silver tube electrode and have been unable to note any difference. We have preferred to use the tube electrode, as it eliminates in large measure the electrical effects due to activation of muscle other than that immediately beneath the contacts, and gives curves which are similar at all positions upon the ventricle. It is also less liable to disturbance from the escape of current used to stimulate the vagus.

The records were taken upon a moving paper camera, and measured by means of a Cambridge type record measurer, the error of which amounts to about 0.003 sec.

#### LEFT VENTRICULAR EXTRASYSTOLES.

##### *The spread of the excitation wave over the surface of the right ventricle.*

Lewis and Rothschild [1914] have offered evidence that when an impulse is liberated in the left ventricle, it passes to the distant portions of the contralateral ventricle by way of the bundle branches. The activation of the opposite ventricle is, however, a race between impulses arriving by the bundle branches and those arriving by muscle spread. It is so important to the interpretation of these present observations that there should be no doubt as to the precise path taken by the excitation wave in such circumstances that we have examined the matter in some detail.

In our experiments we have determined the order of activation of the right ventricle during a series of normal beats and then during a

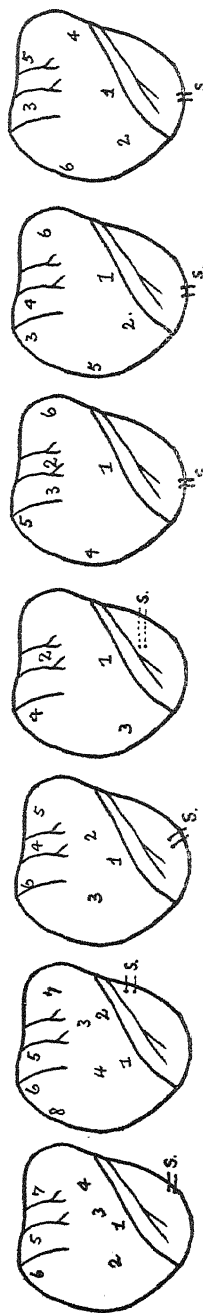
series of extrasystolic beats forced from the left ventricle. Left ventricular extrasystoles were chosen as the right ventricular surface is easy of access experimentally, and the order of activation in this ventricle is well defined owing to the distribution of the right bundle branch of the A.-v. bundle.

The extrasystoles have been obtained by driving the left ventricle by means of rhythmic shocks passed through fishhook electrodes embedded in the musculature, the position of which has been varied in the different experiments. The rhythmic rate used has been slightly above that of the natural heart rate. The spread of the excitation wave over the surface of the right ventricle has been determined by placing the recording electrode at a series of different positions and finding the order, in time, of the activation of these points. This has been done for the normal rhythm and immediately afterwards for the left ventricular extrasystolic rhythm. The time of arrival of the excitation wave has been measured with reference to a standard point in lead II in each instance. With the normal rhythm, the beginning of the R. wave, and with the extrasystolic rhythm, the beginning of the main ventricular deflection have been used.

The results of such experiments are shown in Table I. In this table the excitation wave is considered to reach the earliest activated position at zero time, and the delay in its arrival at the other positions is calculated with reference to this first position. The order of activation is also tabulated. The actual positions chosen and the points of stimulation are indicated in the diagrams (Table I). The measurements show that as far as the normal rhythm is concerned the right central portion is activated first, then the right middle, and finally the portion of the right ventricle close to the A.-v. groove. These results are in agreement with those obtained by Lewis and Rothschild [1914], and are explained by the fact that the right bundle branch through which the normal impulse reaches the ventricle does not break up into the Purkinje network till it reaches a position close to the right central region. The delay in the arrival at points distant from this is due to the time required for the spread through the Purkinje network. It is also to be noted that the actual time values correspond very closely with those observed by Lewis and Rothschild [1914]. When the extrasystolic measurements are considered, it is evident that while the order of activation in the main corresponds with that of the normal rhythm, there are many discrepancies. Although certain of these lie within the limits of experimental error it would be unjustified without further evidence to con-

TABLE I. Spread of excitation wave over right ventricular surface during (a) normal rhythm, (b) a series of left ventricular extrasystoles.

Left ventricular extrasystole				Normal rhythm				Left ventricular extrasystole				Normal rhythm				Left ventricular extrasystole				
Position	Time	Order		Position	Time	Order		Position	Time	Order		Position	Time	Order		Position	Time	Order		
Exp. 3.6.22. Vagus nerves intact.																				
1	0-0000	1		1	0-0000	1		1	0-0000	1		1	0-0000	1		1	0-0054	3		
2	0-0059	2		2	0-0047	3		2	0-0081	2		2	0-0077	2		2	0-0000	1		
3	0-0077	3		3	0-0036	2		3	0-0095	3		3	0-0063	3		3	0-0181	5		
4	0-0084	4		4	0-0051	4		4	0-0190	4		4	0-0154	5		4	0-0168	4		
5	0-0145	5		5	0-0081	4		5	0-0206	5		5	0-0116	4		5	0-0040	2		
6	0-0155	6		6	0-0145	7		6	0-0217	6		6	0-0164	6		6	0-0208	6		
7	0-0161	7		7	0-0138	6		Heart rate } 187			Heart rate } 230			Heart rate } 185			210			
Heart rate } 194			218			Exp. 3.7.18. Vagus nerves cut.			Exp. 3.7.11. Vagus nerves cut.			Exp. 3.8.4. Vagus nerves cut.			220					
per min.						per min.			per min.			per min.			per min.					
Exp. 3.6.28. Vagus nerves cut.																				
1	0-0000	1		1	0-0000	1		1	0-0000	1		1	0-0000	1		1	0-0124	4		
2	0-0081	2		2	0-0015	2		2	0-0011	2		2	0-0067	2		2	0-0000	1		
3	0-0123	3		3	0-0132	5		3	0-0070	3		3	0-0117	3		3	0-0121	3		
4	0-0151	4		4	0-0102	3		4	0-0050	3		4	0-0000	4		4	0-0243	6		
5	0-0162	5		5	0-0112	4		5	0-0082	4		5	0-0153	3		5	0-0049	2		
6	0-0168	6		6	0-0139	6		Heart rate } 175			225			Exp. 3.10.13. Vagus nerves cut.			220			
7	0-0218	7		7	0-0173	7		per min.			per min.			per min.			per min.			
8	0-0246	8		8	0-0222	8		per min.			per min.			per min.			per min.			
Heart rate } 200			230			180			200			200			200			220		
per min.						per min.			per min.			per min.			per min.			per min.		



Diagrams illustrating the positions at which the recording electrode was placed and the point stimulated (S).

clude that the activation of all points in the right ventricle was from impulses which passed down the right bundle branch. If the position of the point of stimulation is such that the central portion of the right ventricle is reached early by muscle spread, the activation of the remainder will follow the normal sequence, for the impulse will spread from this point by the Purkinje tissue as it does normally. To try to obviate this we have stimulated the left ventricle from different positions, namely dorsal and ventral surfaces, and basal and apical regions. In two experiments using the electrodes already described [Dale and Drury, 1932] we stimulated the left ventricle endocardially in the apical region. In none of the experiments was the order of activation identical with that of the normal rhythm. In consequence of this we proceeded to cut the right bundle branch and to compare the time of activation of chosen points in the right ventricle before and after such a section. In these experiments the arrival of the excitation wave was recorded at certain positions during a series of left ventricular extrasystoles, before and after the right bundle branch was cut. The success of this section was shown by the appearance of the typical electrocardiogram in lead II during the normal rhythm, and in Exps. 3.7.18, 3.7.28, and 3.8.4 by the additional observation that, with a normal rhythm, the activation of all the points on the right ventricle was considerably delayed, when compared with measurements obtained before section. The results are tabulated in Table II. The measurements show clearly that we have not been able to find a point of stimulation from which all the positions on the right ventricle are activated by impulses arriving by the right bundle branch. The arrival of the excitation wave at certain positions is definitely delayed by the section: Exp. 3.6.28, positions 1 and 6; Exp. 3.7.18, position 2; Exp. 3.7.28 (*a*), positions 2 and 3; Exp. 3.7.28 (*b*), positions 2, 3, 4, 5 and 6; Exp. 3.8.4, position 1; Exp. 3.10.13, position 5. The delay in other positions is less certain: Exp. 3.6.28, position 2; Exp. 3.7.28 (*a*), positions 1, 4 and 6. But there is no delay in the arrival at one or more points in every experiment: Exp. 3.6.28, position 5; Exp. 3.7.18, positions 1, 3 and 4; Exp. 3.7.28 (*a*), position 5; Exp. 3.7.28 (*b*), position 1; Exp. 3.8.4, positions 2, 3, 4, 5 and 6; Exp. 3.10.13, positions 1, 2, 3, 4 and 6. It can only be concluded that these last positions have been activated by impulses which have travelled through muscle and not through the right bundle branch. As far as our observations go, we feel that it is unlikely that the right ventricle will be activated solely by impulses arriving by the bundle branch, whatever position on the epicardium or endocardium of the left ventricle is chosen

as the point of stimulation. If precise information is desired as to which positions are activated by impulses arriving by the bundle branch, then the branch must be cut in every experiment.

TABLE II. Time in seconds of activation of right ventricular surface relative to main ventricular deflection in lead II during a series of left ventricular extrasystoles (a) before and (b) after section of right bundle branch.

	Position*	Normal	Branch cut
Exp. 3.6.28.	1	0-0205	0-0403
	2	0-0134	0-0175
	5	0-0304	0-0297
	6	0-0396	0-0594
Heart rate 230 per min. Vagi cut.			
Exp. 3.7.18.	1	0-0294	0-0261
	2	0-0209	0-0309
	3	0-0139	0-0136
	4	0-0292	0-0265
Heart rate 225 per min. Vagi cut.			
Exp. 3.7.28.	1	0-0312	0-0261
	2	0-0230	0-0397
	3	0-0226	0-0361
	4	0-0241	0-0293
	5	0-0323	0-0286
	6	0-0293	0-0336
Heart rate 200 per min. Vagi cut.			
Stimulation at vortex of left ventricle. Epicardium.			
	1	0-0211	0-0206
	2	0-0250	0-0427
	3	0-0247	0-0399
	4	0-0264	0-0334
	5	0-0270	0-0354
	6	0-0289	0-0345
Heart rate 200 per min. Vagi cut.			
Stimulation at vortex of left ventricle. Endocardium.			
Exp. 3.8.4.	1	0-0156	0-0251
	2	0-0084	0-0095
	3	0-0305	0-0293
	4	0-0304	0-0332
	5	0-0165	0-0125
	6	0-0405	0-0388
Heart rate 190 per min. Vagi cut.			
Stimulation at vortex of left ventricle. Endocardium.			
Exp. 3.10.13.	1	0-0124	0-0146
	2	0-0000	0-0000
	3	0-0121	0-0149
	4	0-0243	0-0240
	5	0-0219	0-0290
	6	0-0055	0-0083
Heart rate 220 per min. Vagi cut.			
Stimulation at vortex of left ventricle. Epicardium.			

\* See diagrams at bottom of Table I.



*Influence of vagal stimulation upon conduction through  
the bundle branches.*

The observations in the preceding section show clearly that the spread from left to right ventricle is a race between impulses arriving by the right bundle branch and those arriving by muscle. Although it is true that precise information with regard to the influence of vagal stimulation upon conduction is only obtained in those experiments in which the positions activated by impulses arriving by the bundle branch have been determined, the experiments in which no such observations have been made can be usefully employed. Certain positions are apparently activated by impulses arriving by the bundle branches in every experiment, and if it is found that the measurements at all positions are unchanged, then this is presumptive evidence that the vagus nerves have no influence upon branch conduction. If in addition the measurements from the experiments in which the positions activated by branch spread are known, and show no change during the stimulation, then it may be safely concluded that conduction is undisturbed.

In these experiments the arrival of the excitation wave at a point on the surface of the right ventricle was recorded during a series of left ventricular extrasystoles. The right vagus nerve was then stimulated in the neck with a current sufficient completely to arrest the heart; extrasystoles were forced, and the arrival again recorded. The left vagus nerve was then stimulated and another record taken. When acetylcholine was employed, the dose used was enough to arrest the heart completely for 30 sec. when intravenously injected. The electrode upon the heart was then moved to a new position, and the same procedure carried out. A series of positions were tested as quickly as possible one after another, and the measurements are shown in Table III. In some experiments the right bundle branch was cut immediately after these observations had been made and the time of activation of the positions on the right ventricle measured once more. The positions activated by impulses arriving by the bundle branches were thus determined and are shown in heavy type in the table.

The measurements in all the experiments tabulated clearly show that vagal stimulation has no influence upon the spread of the excitation wave. While at some positions a decrease in the time measurement is noted, at others an increase is found. These differences are all small, and fall within the limits of experimental error. The impulses, however, which have travelled to the contralateral ventricle by the bundle

TABLE III. Time in seconds of activation of right ventricular surface relative to main ventricular deflection in lead II during a series of left ventricular extrasystoles (a) normal and (b) during vagal stimulation.

	Position*	Normal	Right vagal stimulation	Left vagal stimulation
Exp. 3.6.22.	1	0.0196	0.0217	0.0237
	2	0.0313	0.0282	0.0312
	3	0.0307	0.0294	0.0317
	4	0.0263	0.0222	0.0269
	5	0.0372	0.0399	0.0353
	6	0.0385	0.0392	0.0388
	7	0.0377	0.0370	0.0333
Heart rate 215 per min. Stimulation at ventral surface of left ventricle. Vagi intact.				
Exp. 3.6.28.	1	0.0205	0.0236	0.0244
	2	0.0220	0.0207	0.0210
	3	0.0337	0.0343	0.0342
	4	0.0306	0.0306	0.0290
	5	0.0317	0.0323	0.0309
	6	0.0344	0.0338	0.0315
	7	0.0378	0.0375	0.0390
	8	0.0427	0.0420	0.0415
Heart rate 230 per min. Stimulation at ventral surface of left ventricle. Vagi cut.				
Exp. 3.7.11.	1	0.0281	0.0327	0.0308
	2	0.0358	0.0357	0.0310
	3	0.0344	0.0307	0.0355
	4	0.0435	0.0413	0.0429
	5	0.0397	0.0393	0.0405
	6	0.0445	0.0437	0.0426
Heart rate 230 per min. Stimulation at lateral surface of left ventricle. Vagi cut.				
Exp. 3.7.28.	1	0.0213	0.0231	0.0237
	2	0.0280	0.0293	0.0261
	3	0.0226	0.0219	0.0224
	4	0.0241	0.0260	0.0271
	5	0.0323	0.0308	—
	6	0.0293	0.0306	0.0302
Heart rate 200 per min. Stimulation at vortex of left ventricle, epicardial surface. Vagi cut.				
	1	0.0211	0.0217	0.0207
	2	0.0250	0.0247	0.0253
	3	0.0247	0.0229	0.0237
	4	0.0264	0.0253	0.0276
	5	0.0264	0.0249	0.0279
	6	0.0289	0.0271	0.0300
Heart rate 200 per min. Stimulation at vortex of left ventricle, endocardial surface. Vagi cut.				
Acetylcholine				
Exp. 3.7.18.	1	0.0305	0.0342	
	2	0.0349	0.0328	
	3	0.0226	0.0243	
	4	0.0319	0.0307	
Heart rate 175 per min. Stimulation at dorsal surface of left ventricle. Vagi cut.				
Acetylcholine				
Exp. 3.8.4.	1	0.0319	0.0325	
	2	0.0265	0.0243	
	3	0.0349	0.0335	
	4	0.0433	0.0415	
	5	0.0305	0.0334	
	6	0.0473	0.0474	
Heart rate 190 per min. Stimulation at vortex of left ventricle, endocardial surface. Vagi cut.				

\* See diagrams at bottom of Table I.

branches, must have passed in addition through muscle and Purkinje tissue from the point of stimulation on the surface of the left ventricle to the recording electrode on the surface of the right ventricle. No conclusions therefore can be drawn in respect of the influence of vagal stimulation upon conduction through the bundle branches until it can be shown that such stimulation has no influence upon conduction through ventricular muscle or Purkinje tissue. Many of the positions on the contralateral ventricle, however, are activated by impulses which have travelled through muscle and, in all probability, Purkinje tissue. As these measurements are unaffected by vagal stimulation (Table III), it is reasonable to conclude that the stimulation has no influence upon conduction through these two tissues. There is, however, additional evidence. One of us [Drury, 1923], using a method which allowed the rate of conduction through ventricular muscle to be measured, was unable to find that vagal stimulation had any influence. Recently we have carried out direct observations upon the effect of vagal stimulation on conduction through ventricular muscle and Purkinje tissue combined. The method used has been to test the rate of conduction through a long stretch of right ventricular muscle. If a sufficient length of muscle is taken, owing to the high rate of conduction through Purkinje tissue compared to that through muscle, the impulse reaches the distant parts more quickly by passing inwards to reach the Purkinje tissue and outwards again through muscle, than by travelling through muscle alone [Lewis and Rothschild, 1914]. This pathway will be chosen if the distance is more than twice the thickness of the muscle wall. We have therefore stimulated the right ventricle and placed one pair of recording electrodes close to the point stimulated, and another pair at a distance away greater than twice the thickness of the muscle. In such circumstances we are unable to find that vagal stimulation has any influence upon the rate of conduction.

From these experiments it is safe to conclude therefore that vagal stimulation does not influence either conduction through ventricular muscle and Purkinje tissue or through the bundle branches. There is one position in the path travelled that we have been unable to test. We have no evidence of the influence of vagal stimulation upon the crossing of the impulse from the left to the right bundle branch. It might be argued that the vagal effect was to slow conduction in the bundle and to speed up the crossing of the impulse from the left to the right bundle branch. The fact that our measurements remain undisturbed renders such an explanation extremely unlikely, for the two effects would scarcely counterbalance one another so precisely.

During a series of left ventricular extrasystoles the ventricular complexes in lead II are unchanged by vagal stimulation; they are, in fact, superimposable in every way. This, in itself, is evidence that there has been no disturbance of conduction. Careful measurement of the extrasystolic curves from lead II before and after right bundle branch section shows that the duration of the complex is usually increased by about 0.01 sec., the Q.R.S. group being slightly widened. The activation of those positions supplied by impulses travelling by the bundle branches is delayed by about 0.01–0.02 sec. when the right bundle branch is sectioned, so that the observed increase in duration of the complex agrees very closely. In some experiments, however, we can detect no appreciable change in the curves before and after branch section. Although, therefore, the fact that vagal stimulation has no influence upon lead II may be taken as evidence that the conduction through the bundle branches is undisturbed, it is to be noted that the change produced by section of the right bundle branch is often very slight.

*The region at which the impulses pass from the left  
to the right branch of the A.-V. bundle.*

When an extrasystole is forced from the left ventricle and the excitation wave travels to the right ventricle by means of the bundle branches, it must cross at some region from the fibres which lead from the left ventricle to those which lead to the right ventricle. Experiments of the type already described give some idea of the region at which impulses can so pass.

In Exp. 3.7.28 the animal exhibited a condition which is occasionally seen. When the left ventricle was driven by rhythmic shocks at a rate slightly above that of the natural rhythm the impulse completely failed to pass to the auricle, complete retrograde heart block being present. In addition, when an auricular beat occurred at a suitable moment during the extrasystolic rhythm, it passed readily to the ventricle, and gave rise to a supraventricular response, thereby disturbing the extrasystolic rhythm. There was no indication of any degree of forward heart block. This disturbance to the extrasystolic rhythm was obviated in the experiment in question by driving the ventricle and auricle simultaneously.

When the time measurements in the experiment in question are considered, it is seen that the excitation wave arrives at the surface of the right ventricle in much the same time as in experiments in which no retrograde heart block was seen (Table III). This is true for positions

which are known to have been activated by excitation waves passing through the right bundle branch. It may therefore be concluded that the impulse liberated in the left ventricle passed through the bundle branches without any abnormal delay, and crossed below the place at which the retrograde heart block occurred.

In Exp. 3.7.11 the A.-v. bundle was cut, and complete heart block resulted. At post-mortem, the cut was found to be 2 mm. deep and 5 mm. long and was situated in the long axis of the heart, about 1.0 cm. to the left of the opening of the coronary sinus, and at the same level. The activation of positions on the right ventricular surface was uninfluenced by the section (Table IV). It is not possible to say which

TABLE IV. Time in seconds of activation of right ventricular surface relative to main ventricular deflection in lead II during a series of left ventricular extrasystoles. Influence of (a) section or clamp of main bundle and (b) section of right bundle branch.

	Position*	Normal	Main bundle destroyed	Branch cut
Exp. 3.7.11.	1	0.0368	0.0376	—
	2	0.0357	0.0311	—
	3	0.0242	0.0294	—
	4	0.0417	0.0424	—
		Heart rate 230 per min. Vagi cut.		
Exp. 3.8.4.	2	0.0319	0.0306	0.0401
		Heart rate 190 per min. Vagi cut.		
Exp. 3.10.13.	3	0.0209	0.0213	0.0290
		Heart rate 220 per min. Vagi cut.		

\* See diagrams at bottom of Table I.

positions were activated by impulses passing through the right bundle branch, as this was not cut in this experiment. It is reasonable from our observations to assume that some of the positions were so activated. As all the time measurements remain undisturbed, it is evident that the passage from the left to the right bundle branch occurred below the section.

In Exps. 3.8.4. and 3.10.13 the bundle was compressed with a clamp and complete heart block produced. At post-mortem bruised areas about 0.8 cm. long and 0.3 cm. wide, roughly corresponding to the size of the blade of the clamp, were found situated about 3 and 5 mm. respectively to the left of the opening of the coronary sinus and at the same level. The times of activation of positions on the right ventricle, which were found later by right bundle branch section to have received their impulses by the right bundle branch, were undisturbed by this procedure (Table IV). It is evident also from these experiments that the impulse must have crossed from the left to the right bundle branch at a position

close to the bifurcation, as although the main pressure of the clamp is exerted at its point of application, a definite though lesser pressure must be exerted a distance away. The precise position at which an impulse can cross is not to be decided by such experiments, but it is certainly well below the A.-v. node itself.

#### DISCUSSION.

Although it is possible, in some of the recorded instances, that the aberrant ventricular beats which are in apparent response to supraventricular stimuli are in reality ventricular escaped beats which coincide in time with the expected supraventricular beats, it is not easy to offer this explanation in all cases. Moreover, aberrant beats are seen when the septal branch of the left coronary artery is ligated [Kahn, 1911; Lauterbach, 1928]. We feel therefore that supraventricular stimuli are capable of giving rise to aberrant ventricular complexes under certain conditions. The simple explanation suggested by Einthoven and Wieringa [1914], namely, that the vagus nerves influence conduction in the bundle branches, and that their influence is sometimes exerted more strongly upon one branch than the other, so that the impulses reach one ventricle before the other and give rise to aberrant ventricular beats, is not supported by our observations. In fact our results show so clearly that stimulation of the vagus nerves, either by faradization in the neck or by the injection of acetylcholine, has no influence upon conduction in the bundle branches, that the explanation can no longer be accepted.

Two other possible explanations suggest themselves. The first is that the supraventricular impulses pass into right and left bundle branch tracts at a position in the bundle below which no crossing is possible, and this differentiation occurs at a region where vagal stimulation slows down conduction. If the vagal action is unequal, aberrant ventricular beats will occur. On this theory also, the impulses which pass upward through one branch will not be expected to cross to the other until they reach the region above that at which differentiation into two separate tracts occurs and where the vagus nerves slow down conduction. We are unable to find any evidence that the impulses are slowed. Moreover, we find that those impulses passing upward through the left bundle branch can cross to the right at relatively low levels. On these grounds we feel this explanation is untenable unless it is accepted that impulses travelling from the auricle to the ventricle are unable to cross at the same low levels as those which cross from branch to branch.

The second is that the junctional tissues (*i.e.* node, bundle and branches) between auricle and ventricle conduct with a decrement.

This is evidence that conduction with a decrement occurs in damaged auricular muscle [Drury, 1925; Drury and Andrus, 1924]. It also occurs during the period of recovering conduction in normal auricular muscle [Drury and Regnier, 1929]. It has not been noted in normal auricular muscle after full recovery of conduction has taken place, as the method of experiment available does not allow of either proof or disproof. Schmitt [1928] has demonstrated decremental conduction in strips of turtles' ventricular muscle poisoned with potassium chloride. Decremental conduction through the junctional tissue is strongly suggested by the work of Lewis and Master [1925] on heart block produced by vagal stimulation, asphyxia and quinidine. There is considerable evidence that decremental conduction can occur over long stretches of cardiac tissue in contradistinction to nerve where it is limited to very short stretches [Edwards and Cattell, 1927].

Conduction through a decremental area can be disturbed in two ways, the gradient of decrement may become more steep or the strength of the impulse entering the region may be weakened. Schmitt [1928] has shown if an impulse entering a region of constant decrement is weakened, it travels with greater difficulty. Vagal stimulation does not disturb conduction through the bundle branches so that it must be concluded that it does not change the gradient of decrement in this region. If, however, it increases the gradient of decrement at the higher levels, or decreases the impulse strength (*i.e.* the ability to pass through a region of disturbed conduction), then the impulse will be below normal strength when it arrives at the bundle branches. A local condition constantly present, and not connected with vagal stimulation, may steepen the gradient more in one branch than the other, so that the weakened impulse will be relatively more delayed in one branch than the other or even run to extinction, while a normal impulse would pass without difficulty<sup>1</sup>.

In those conditions in which complete vagal heart block is present, and the idioventricular beats are abnormal, we feel that the more likely explanation is that the impulses arise in the bundle above its bifurcation and are subject to decremental conduction rather than that they arise in a bundle branch.

<sup>1</sup> Recent observations made by us lend support to this conception.

## SUMMARY.

1. The order of activation of the right ventricular surface during a series of left ventricular extrasystoles is similar to but not identical with that found during a normal rhythm.

2. Wherever the left ventricle is stimulated some positions on the right ventricle are activated by impulses which travel through muscle, others by those which travel through the bundle branches.

3. Vagal stimulation has no influence upon the spread of these impulses, whether they are travelling through muscle or through the bundle branches. It is concluded therefore that vagal stimulation does not disturb conduction in the branches of the A.-V. bundle.

4. The relation of this finding to the aberrant ventricular beats seen during vagal stimulation is discussed.

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## THE BIOLOGICAL SIGNIFICANCE OF THE LINKAGES IN ADENOSINE TRIPHOSPHORIC ACID.

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For some years before 1929 interest had been aroused in the adenyl group of compounds by the recognition of the important part played by them in the glycolytic processes of muscular activity. Since then, additional significance has been attached to them because of the demonstration by Drury and Szent-Györgi [1929] of certain characteristic biological actions. After having studied the activity of simple saline extracts of cardiac muscle, they isolated from the trichloroacetic acid extract of bullock's heart a crystalline substance which their analysis showed to be adenylic acid. This acid was undoubtedly derived from the adenosine triphosphoric acid (a.t.p.) present in the original extract.

They found that adenylic acid produced a typical heart block in the guinea-pig, dilatation of the coronary arteries, lowering of blood-pressure in the dog, and diminution in the amplitude of contraction of isolated strips of intestine.

Lindner and Rigler [1930] found that extracts of the sinus, node of Tawara, and bundle of His from the calf's heart, prepared in a manner not specified, stimulated an hypodynamically acting frog's heart. They attempted to fractionate their extracts, and eventually prepared a substance which dilated the coronary arteries in the mammalian heart, and had no effect on the frog's heart. This substance they found to contain a pentose and a purine base.

Work of this nature, including that of Rigler and Schaumann [1930] and of Rothmann [1930], began to make it clear that the biological activity of many of the tissue extracts which had been studied by earlier workers was really due to their adenosine content.

Zipf [1930] showed clearly that the depressor substance which can be isolated from defibrinated blood, and which had been described by Freund [1920] as "Frühgift," was adenylic acid.

Bennet and Drury [1931] further investigated the production of heart block in the guinea-pig by injection of the adenylic compounds, and concluded that the amounts required to produce block were independent of the presence of other substances, and therefore might be used as a method for the biological assay of the adenylic acid present in tissue extracts. Adenylic acid was thus recognized in nearly all tissues, especially in heart and voluntary muscle.

Wedd [1931] investigated the action of adenosine and allied compounds on the coronary flow of the perfused rabbit's heart. He noticed the difficulty of correlating this action with that upon the musculature of the heart, which he concluded must be of a different nature. The maximal dilatant effects could be obtained with amounts which caused little or no cardiac slowing. He also noticed that adenosine was a more powerful coronary dilator than adenylic acid obtained from muscle or yeast.

Hochrein and Keller [1931] compared various tissue extracts by their effects on the blood-pressure and the electrocardiogram, and concluded that the active principle of most of them was the same, but no details were given regarding the chemical nature of the active substances.

Subsequently, Drury [1932 *a*] revised his view that deamination was the essential factor in the activity of the adenylic compounds, and he attempted to explain the effects as partly due to the presence of phosphate radicles in the molecule. He found, for example, that orthophosphate increased the amplitude of the mechanogram, while pyrophosphate first decreased and then increased it. Both forms of inorganic phosphate, however, reduced the coronary flow. Drury therefore supposed that the beat might be influenced by (1) the adenylic complex acting on the coronary vessels, and (2) the phosphate radicles acting on the musculature. This, however, is not sufficient to account for the fact that adenylic acid and even adenosine may produce heart block indistinguishable from that caused by a.t.p. Moreover, in producing effects with inorganic phosphates, Drury had to employ doses far outside the range of those used in the case of the adenylic derivatives, so that the effects are scarcely comparable.

Ostern and Parnas [1932] estimated the adenosine content of various tissue extracts by perfusion of the frog's heart. They stated that a.t.p. was three times as active as adenosine, judging by the time taken for heart block to develop. Deuticke [1932] compared the activities of the adenylic compounds on the virgin guinea-pig's uterus. Following a short latent period, contraction was caused by adenosine, adenylic acid, and a.t.p. as well as by yeast adenylic acid and a related nucleotide

recently isolated by Embden from the heart. He emphasized that the maximum height of contraction was reached more rapidly with substances which contained more phosphorus in their molecule.

Marcou [1932] examined the cause of the lowering of blood-pressure by adenosine, and found that it was evidently largely due to dilation of intestinal and peripheral vessels. The volumes of the spleen and kidney were at the same time diminished.

Gaddum and Holtz [1933] tested adenosine, adenylic acid, and a.t.p. on the vessels of the lung. They found the cat more sensitive than the dog: in both, constriction occurred, with fall in lung volume.

There is, therefore, a considerable accumulation of knowledge about the biological actions of adenylyl compounds, which ought to make possible a definite statement as to their rôle in the activity of any given tissue extract. Unfortunately, the substances tested by most of the authors quoted have been obtained from widely divergent sources, and it is difficult to correlate the changes which occur in the biological activity of the adenylyl compounds with variations in their chemical constitution. For example, Drury suggested that ease of deamination is not the essential factor in their biological activity from a comparison of yeast adenylic acid and muscle adenosine. The former cannot be deaminated by enzymes, while the latter can, although only with difficulty, and under special conditions. In several of the papers the exact source of the compound tested has not been stated.

A series of experiments was therefore carried out using the various derivatives from the same mother substance, the naturally occurring adenosine triphosphate of skeletal muscle. This substance was prepared from rabbits' muscle as a calcium salt. The distribution of the phosphorus per mg. dried salt was as follows:

Free (inorganic) phosphorus	Nil
Labile P hydrolysable in 10 min.	0.096 mg.
Total P liberated by total hydrolysis	0.143 mg.

The ratio of labile to stable phosphorus is the theoretical one of 2 to 1 and the N : P ratio was found to be the theoretical one (5 : 3).

As the changes most easily produced in the natural nucleotide are deamination and the removal of the two labile phosphoric acid molecules, it is necessary to compare the biological activities of adenosine—and inosine—triphosphoric acids (a.t.p. and i.t.p.), and of adenylic and inosinic acids respectively. It is evident, however, from the earlier work, that so long as the pentose linkage with adenine is retained, certain of the

characteristic effects produced by the adenylic derivatives are still obtainable even when the ester linkage between the sugar and phosphoric acid has been broken. Although doubts have been expressed regarding the possibility of the removal of  $\text{NH}_2$  from a.t.p., it is possible to obtain i.t.p. by nitrous acid deamination. The body so formed has a 4N : 3P ratio and retains the two labile phosphoric acid molecules. Adenylic and inosinic acids may be readily obtained by weak alkaline hydrolysis of a.t.p. and i.t.p. respectively. Adenosine may be obtained in the usual way by alkaline hydrolysis at  $170^\circ$ , while the purine-pentose linkage can be readily broken by acid hydrolysis.

For purposes of injection, salts were used which were obtained either as hydrolysed solutions of a.t.p., analysed for their content of the derivatives, or in the dried purified form, made up to the required watery dilution. In the former case, the procedure was briefly as follows.

A weighed amount of the pure a.t.p. was suspended in the hydrolysing solution, and heated in sealed glass tubes. When it was desired to obtain adenylic acid as the sole derivative of alkaline hydrolysis, the water was made sufficiently alkaline to neutralize the two labile phosphoric acid molecules which would be set free. The tubes were then heated at  $100^\circ \text{C}$ . for 2 hours. The hydrolysed solution showed on analysis that the rise in inorganic phosphate was fully accounted for by the liberation of the labile  $\text{H}_3\text{PO}_4$ , without encroachment on the stable  $\text{H}_3\text{PO}_4$  of the adenylic acid molecule.

It was thus easy to obtain the adenylic acid content of the solution, which also contained the labile  $\text{H}_3\text{PO}_4$  of the original a.t.p. in the form of inorganic phosphate. In experiments carried out for the purpose, it was found that this inorganic phosphate was quite inactive in the amounts in which it occurred in the hydrolysed solutions.

When it was desired to obtain adenosine by hydrolysis, a weighed amount of a.t.p. suspended in 16 p.c. ammonia was heated at  $170^\circ \text{C}$ . for 2 hours. Analysis showed that this procedure set free all three  $\text{H}_3\text{PO}_4$  molecules and, since the glucoside linkage remains unbroken under these conditions, it could reasonably be assumed that the solution contained only adenosine plus inorganic phosphate.

The ammonia used in the solutions was removed by aeration, and the reaction was brought to  $\text{pH}$  7.3 by a few drops of dilute hydrochloric acid.

Inosine triphosphate was prepared by deamination of a.t.p. with nitrous acid, and the doses referred to in the text were the weights of pure dry sodium salt used for injection. The salt may have had some very

slight admixture with non-deaminated a.t.p., but the ratio of N to P was approximately 4 : 3 instead of 5 : 3.

Inosinic acid was also used after separation as a pure barium salt, prepared in the laboratory after the method of Ostern. For some confirmatory tests, a sample of pure dry barium inosinate, kindly sent by Dr Levene, was also used. In all cases the barium was removed before the salt was used biologically, the sodium salt being that employed. Adenosine was also tested in the form of "Lacarnol," kindly supplied by Messrs Bayer. Contrary to the statement of Rothmann [1930] we found on repeated analysis that the lacarnol in our possession was phosphorus-free, and therefore could not be a nucleotide. Whatever other components it may contain, adenosine can be recovered from it in practically pure form.

The actions of the salts on different test objects will now be described in order.

#### BLOOD-PRESSURE.

##### (1) *Adenosine triphosphate and inosine triphosphate.*

The rabbit was largely used, but occasionally also the cat, the actions in the two animals being very similar. The blood-pressure was measured by a cannula tied in the carotid artery, and injection was made slowly

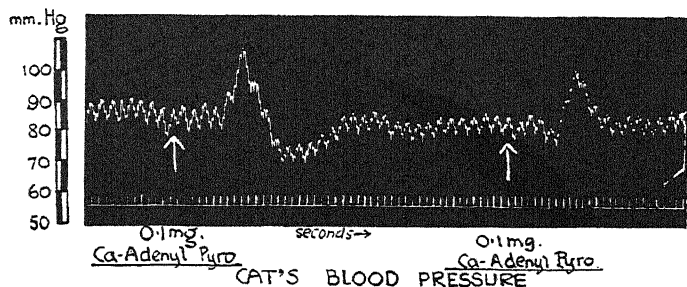


Fig. 1. Effect of injection of 0.1 mg. adenosine triphosphate on cat's blood-pressure. On repetition of the injection, the blood-pressure rise alone is seen.

into a cannula in the jugular vein. The time taken to inject was gauged so as not to affect the blood-pressure, and was about 7 sec.

The usual fall in blood-pressure follows injection of both these substances, but so long as the labile phosphoryl linkage is retained in the molecule, there is frequently to be seen a transient rise in pressure preceding the fall. These effects persist after atropinization.

A typical example may be given (Fig. 1). On injection of 0.1 mg. of adenosine triphosphate into the jugular vein of a cat after a latency of 7 sec., there was a rise in pressure of 20 mm. of mercury, followed at once by a fall of 18 mm. below the normal level. When a second injection is given rapidly after the first one, the rise in blood-pressure alone may be obtained.

The deaminated derivative is much less active, and doses five to ten times as great as those used with the mother substance are necessary to produce the effects. But the transient rise and subsequent fall are both evident when sufficient amounts (say 1 mg. of the dried salt) are injected.

#### (2) *Adenylic acid and inosinic acid.*

The well-known fall in blood-pressure was seen following injection of adenylic acid, but rarely with any evidence of the initial rise, which generally appears to indicate the presence of the labile phosphate in the molecule. After deamination the same decrease in activity is to be observed as in the case of the mother substance. Inosinic acid resembles adenylic acid in producing only the fall in pressure. The most inactive specimen of inosinate was that prepared by Dr Levene, which only produced a very slight fall in doses of 2 mg. Bennet and Drury [1931] described inosinic acid as quite inactive in their experience.

#### (3) *Adenosine.*

The typical fall in blood-pressure was obtained after injection of 0.1 mg. of pure adenosine and, judging by the duration of the fall, it appeared to be the most active of all the non-deaminated derivatives.

#### (4) *Adenine and hypoxanthine.*

These were tested after neutralization of the acid hydrolysed solutions of adenosine and inosine triphosphate. They were always found to be inactive.

### HEART.

Rabbits' hearts were perfused using a modification of Gunn's apparatus, the coronary flow being estimated by allowing the fluid to fall into a bucket which tipped when it contained 1.5 c.c. In order to construct suitable graphs to show the relative activities of the various bodies as coronary dilators, the reciprocal of the time taken for 1.5 c.c. to perfuse was used as a measure of the rate of flow. The rate measured before injection of the drug was taken as 100, and the increase in this rate during

every second following injection was calculated as a percentage. The figures so obtained were plotted in the manner seen (Fig. 2), and all the graphs shown were constructed from the average figures of numerous experiments.

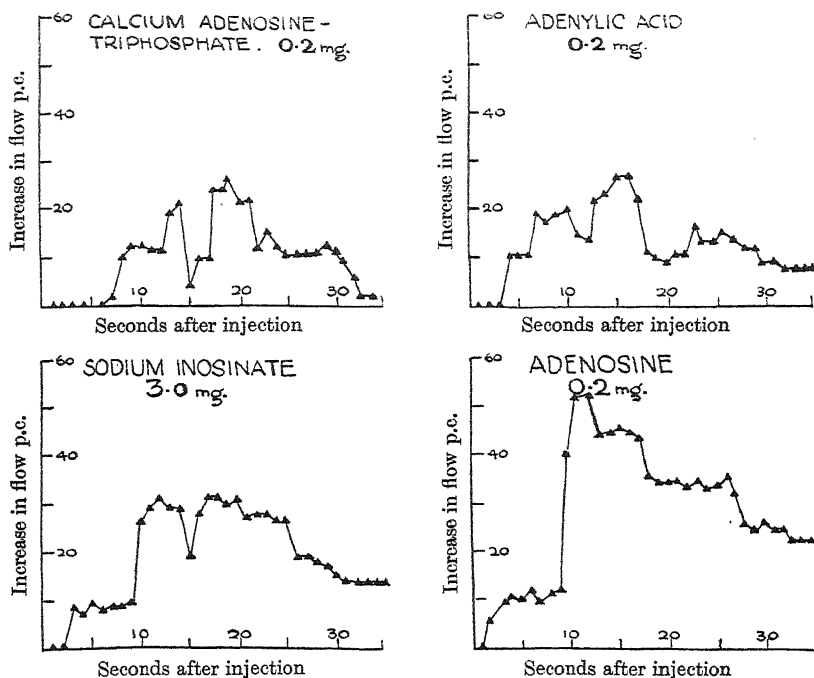


Fig. 2. Graphs showing the percentage increase in coronary outflow in perfused rabbits' hearts after injection of adenylic derivatives.

(1) *Adenosine triphosphate and inosine triphosphate.*

When 0.2 mg. of a.t.p. was injected into the side-piece of the cannula there was a latent period of 7 sec., after which an increase in the flow commenced which reached its maximum in 19 sec. The maximum increase was 26 p.c. above the normal, and in 33 sec. the flow had almost returned to normal. Very similar figures were obtained, using i.t.p., except that about ten times the dose was necessary to produce the effect. The graph is not shown because only five experiments were carried out with this drug.

Some workers have described improvement of the action of the heart by the adenylic compounds, while others only mention inhibition. This

appears to be because the action on the musculature and conducting system is really always depressant, while the improvement in the beat is secondary to the increased coronary flow. The reasons on which this statement is based are the following.

(1) In the mammalian heart, since the dose required to produce blocking of conduction is greater than that which will dilate the coronary vessels, it is sometimes possible to obtain an improvement in amplitude with a small dose, and the reverse effect with a larger dose. This is seen in Fig. 3, where injection of 0.1 mg. of adenosine triphosphate induced a

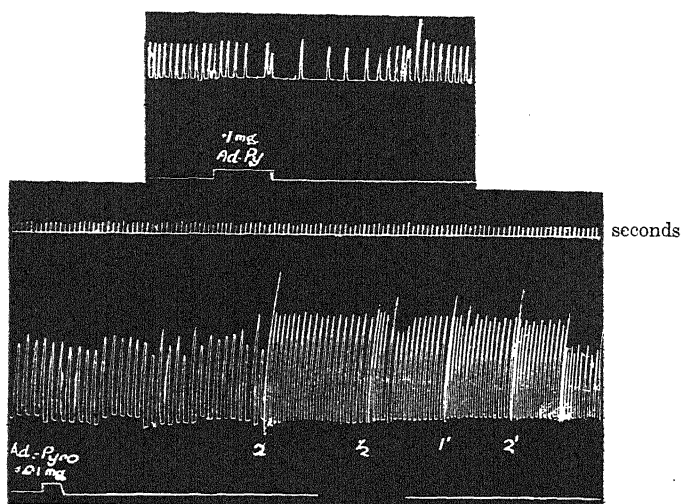


Fig. 3. 0.1 mg. adenosine triphosphate produced "heart block," while in the same heart 0.01 mg. dilated the coronary vessels and improved the beat.

"heart block," while in the same heart a dose of 0.01 mg. dilated the coronary vessels and improved the beat. Unfortunately a record of the coronary output was not kept in this case. Some of the improvement in amplitude in this record is doubtless due to the increased rate of beating, as pointed out by Dale.

(2) An increase in amplitude of the beat was only seen after the coronary outflow had been increased. The improvement followed a short latent period during which there was no coronary dilatation, or the beneficial effects of the dilatation had not been felt. During this period the "blocking" effect might appear (Fig. 4).

(3) In a heart where the coronary arteries were already fully dilated



no increase in amplitude of the beat has been seen to follow injection of the adenylyl compounds.

(4) In the course of a large number of experiments in which the frog's heart was perfused with the Straub-Fühner cannula the adenylyl compounds were always seen to produce interference with conduction of the beat, and diminution in amplitude, and never an improved action of the heart.

It may here be mentioned that a.t.p. was the most active of the adenylyl group in producing heart block in the frog's heart.

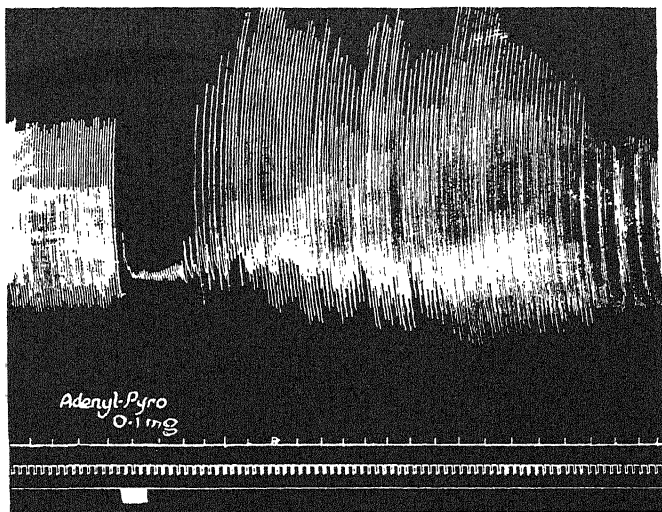


Fig. 4. The typical effect of adenosine triphosphate on the perfused mammalian heart.

The typical action of a.t.p. on the mammalian heart is seen in Fig. 4. This shows the combination of the blocking which is a direct action on the conducting system, and the improvement in the beat following the increased coronary flow. In this case there is very little alteration in the rate of beating, and any increase which there may be is not sufficient to account for the improved amplitude.

A similar picture, using ten times the dose of a.t.p., is given by i.t.p.

#### (2) *Adenylic acid and inosinic acid.*

The dilatation of the coronary vessels produced by adenylic acid resembles that caused by a.t.p. (Fig. 2), except that the increase in flow began 3 sec. earlier, and after 35 sec. was still 8 p.c. above the original

value. This may be taken as evidence of somewhat greater dilatant activity.

The graph obtained with sodium inosinate is very similar, but the effective dose was 3 mg., while 0.2 mg. was the dose employed with the non-deaminated compounds.

Adenylic acid and inosinic acid both produce heart block in the perfused amphibian and mammalian heart, but the loss of the labile phosphate from the molecule causes a loss of activity in this respect, as compared with a.t.p. and i.t.p. The criteria of this were the duration of the block in the rabbit's heart, and the time taken for the block to develop in the frog's heart.

(3) *Adenosine.*

Removal of both labile and stable phosphate groups from the molecule of adenosine triphosphate still further increases the dilatant activity upon the coronary arteries. Thus the most active of our series was found to be the adenosine prepared by alkaline hydrolysis (Fig. 2). The increased flow begins after 1 sec., reaches 52 p.c. above the normal in 12 sec., and after 35 sec. is still 22 p.c. above normal.

Using the same criteria as in the case of adenylic acid, adenosine appears to be the least active of the series in producing heart block. If its action in the intact animal be similar, this may help to explain the clinical popularity of adenosine, since it has the greatest margin of safety between the dose which dilates the coronary vessels and that which produces heart block. Nevertheless, Honey, Ritchie and Thompson [1930] have described dangerous interference with conduction in the human heart following intravenous injection of 50 mg. of adenosine.

(4) *Adenine and hypoxanthine.*

These were quite inactive on amphibian and mammalian hearts.

ISOLATED SMALL INTESTINE.

(1) *Adenosine triphosphate and inosine triphosphate.*

A.t.p. has a unique action on the small intestine which does not appear to have been mentioned in the literature. Like the other adenyl compounds it produces a fall in tone in concentrations as low as 1 in 500,000. But in many cases, and specially if the tone be initially low, it produces and maintains a subsequent rise in tone (Fig. 5). This increase in tone is sometimes very striking, and has been seen in a gut which showed no sign of life until the a.t.p. was added to the solution.

During the action of the drug the rate of contraction is not affected and, with the general increase in tone, the amplitude of the single contractions is decreased. Usually slightly higher concentrations (say 1 in 50,000) are required to bring out the tonic effect.

I.t.p. inhibited the gut in concentrations ten times as great as those in which the mother substance was active, but was never seen to produce increase of tone.

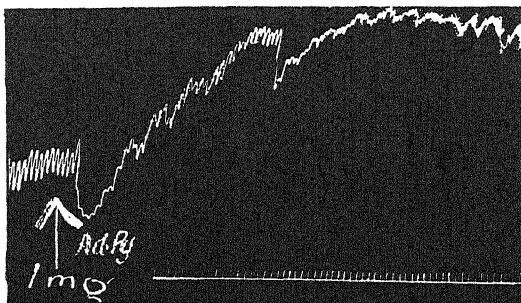


Fig. 5. Increased tone caused by adenosine triphosphate acting on small intestine in concentration of 1 in 50,000.

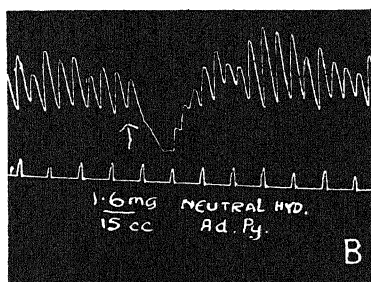
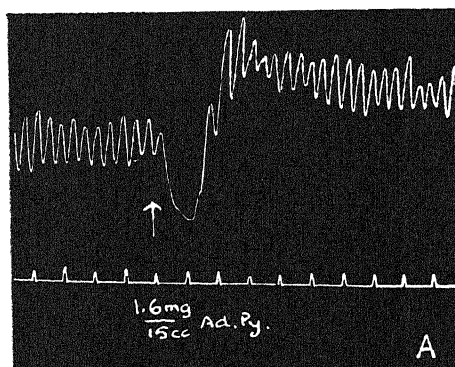


Fig. 6. A. Shows the inhibition followed by increase in tone of the intestine caused by a.t.p. in a concentration of about 1 in 10,000. Time=5 sec. B. Shows how after hydrolysis in neutral solution (adenylic acid being formed) only the inhibition appears.

## (2) *Adenylic acid and inosinic acid.*

Removal of the labile phosphate from the molecule of a.t.p. leaves the power of inhibiting the gut slightly weaker, while the subsequent increase in tone is no longer to be observed except very rarely with larger

doses. Comparison of Fig. 6 A and B shows how hydrolysis brings about this alteration in activity.

Inosinic acid gave results similar to adenylic acid in doses five times as great, only the inhibition of the gut being seen.

(3) *Adenosine.*

Adenosine inhibits the gut in concentrations of 1 in 50,000. It is the least active of the non-deaminated adenylic derivatives in this respect. It has only once been seen to produce increase in the tone of the gut, and that was with an unusually excitable specimen.

(4) *Adenine and hypoxanthine.*

These substances showed no action on the gut.

UTERUS.

The isolated virgin guinea-pig's uterus was used after the technique of Sawasaki [1925], as recommended by Deuticke [1932].

(1) *Adenosine triphosphate and inosine triphosphate.*

With a tension of 6 g. on the cornu, a.t.p. produces tonic contraction in concentrations of 1 in 500,000, or even less. The action does not appear to be as completely reversible as Deuticke suggests. Often the cornu starts to contract at a high level of tone after the specific action of the substance has passed off, and it is difficult to abolish these rhythmic contractions even by washing out the fluid. Since the uterus must be quiescent before a test can be made, the contractions may make it impossible to use the same preparation for testing a second specimen.

I.t.p. must be present in ten times the concentration of a.t.p. before it can produce increase in tone.

(2) *Adenylic acid, inosinic acid, and adenosine.*

Removal of phosphate from the molecule decreases the activity of these compounds on the uterus, so that a.t.p. is the most active of the non-deaminated series, and adenosine the least active.

Inosinic acid appears to have about one-tenth the activity of adenylic acid on this test object.

(3) *Adenine and hypoxanthine.*

These showed no activity on the uterus.

Some experiments were made in order to discover whether the adenylyl compounds are freely dialysable in active form from the tissues where they naturally occur. Analyses of the fluids inside and outside a collodion tube containing a saline muscle extract, which had been allowed to dialyse against a Ringer-Locke solution for varying periods, showed that equilibrium was reached in the case of the adenylyl compounds in about 2 hours, and that this time was unaffected by the presence of the colloid material in the muscle extract. Of course, if the glycolytic ferment were not inactivated by boiling, the ordinary enzymic changes would go on in the extract, and the a.t.p. would lose at the least some of its labile phosphate before it could escape into the outer fluid.

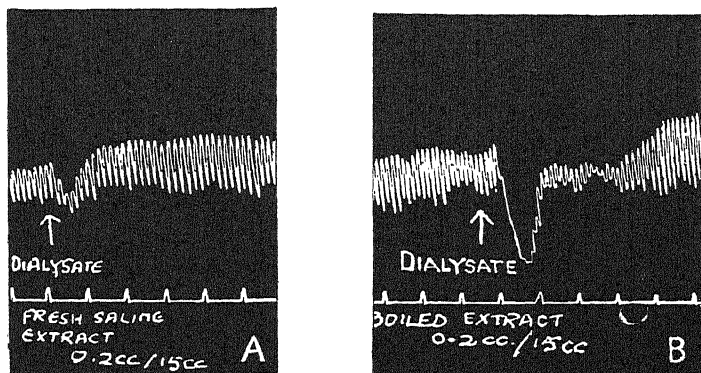


Fig. 7. A. Shows the effect of adding 0.2 c.c. of a 2 hours' dialysate of fresh saline extract of rabbit's muscle to 15 c.c. Ringer-Locke solution in which intestine was contracting. Time = 5 sec. B. Shows the effect of adding 0.2 c.c. of a dialysate of a similar extract in which ferment changes had been inhibited by boiling.

Note the much stronger activity seen in B.

The figure shows that, in the extract in which ferment action had been checked by boiling, the adenylyl compounds can escape in biologically active form (Fig. 7). It is therefore possible that they may do so in the living animal, and, as we have pointed out, Zipf [1930] has shown clearly that the action of defibrinated blood plasma in lowering blood-pressure is due to its content of adenylic acid, which must consequently be always present there in small quantities during life.

The question thus arises as to whether the biological activities which the adenylyl bodies show under experimental conditions have any counterpart in their normal activity in the living animal.

At the present time we can do no more than guess at the answer to this question. If we consider broadly the actions of all these compounds, two

facts are made fairly clear. In the first place complete loss of biological activity does not occur until the pentoside linkage in the molecule has been broken down. This has been pointed out recently by Ostern and Mann [1933], who note that the presence of phosphate in the molecule is not essential for the possession of biological activity. These authors say, however, in common with most workers, that deamination of the substances inactivates them completely. This we do not believe to be always the case. In fact, as far as we can see, the adenylyl compounds never become completely inactive in the tissues, since deamination and removal of the phosphate is the greatest amount of destruction which they are likely to undergo.

But it is true in the second place that these compounds do suffer changes which very markedly alter their biological activity, and these changes occur constantly during muscular activity. The addition of phosphate to the molecule increases the activity of the compounds where tonic and muscular effects are concerned. Examples of this are the effects seen on the heart muscle, the intestine, and the uterus.

On the other hand, the removal of phosphate from the molecule seems to increase the activity of the substances as regards ability to dilate blood vessels. This has been seen in the case of the general blood-pressure, and most strikingly in that of the coronary vessels.

Possibly the tonic properties of the a.t.p. are made available for the muscle during anabolism and rest, while the early removal of the labile phosphate from its molecule during activity brings into play the more powerful dilatant actions of adenylic acid and adenosine. We should thus have a local adjuvant to the vaso-dilatation of activity in the normal breakdown products of metabolism.

It would seem, by reason of the extreme rapidity of all the biological actions, that the effects are really produced by alterations in the colloids at the surfaces of the cells concerned. A hundredth part of a milligramme of a.t.p. which succeeds in dilating one of the coronary arteries as it is whirled past at high pressure in a great volume of fluid has certainly not had time to take part in an elaborate metabolic process. It is this extraordinary rapidity of response, as well as of chemical change, which makes the approach to the problem so very difficult.

#### SUMMARY.

1. Adenosine triphosphate, the naturally occurring adenylyl compound, is more active than any of its derivatives in producing interference in conduction in the heart, and increased tone in the isolated virgin guinea-

pig's uterus. The removal of phosphate from its molecule does not lessen its activity so markedly as deamination.

2. A.t.p. has a tendency, which rarely appears in any of its derivatives, to increase the tone of the small intestine, after the preliminary depression which is also produced by the other active adeny compounds. Deamination, and, to a less extent, the successive breaking off of the labile and stable phosphate linkages, lessen its depressant activity on the intestine.

3. Removal of phosphate from the molecule of a.t.p. increases its ability to lower blood-pressure and to dilate the coronary vessels. Thus the order of activity of the adeny series is adenosine, adenylic acid, and a.t.p. Deamination greatly lessens, but does not destroy, this vasodilant power.

4. A combination of the heart block and coronary dilation may give an explanation of some of the inconsistent behaviour of the so-called "heart hormones."

5. Biological activity finally disappears from the compounds when the pentose is split off from the purine base.

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## THE EFFECT OF INSULIN AND OTHER FACTORS ON IODO-ACETATE HYPERGLYCÆMIA.

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IN a preliminary communication [1932] I showed that iodo-acetic acid, in toxic doses, caused a fairly pronounced hyperglycæmia in rabbits. This observation has also been made by Neuss [1931] and Hikiji [1932]. In addition, I observed that iodo-acetate was antagonistic in its action to insulin. The following experiments have been carried out in an attempt to elucidate the basis of this effect.

### EXPERIMENTS ON THE INTACT ANIMAL.

In all cases the acid used was recrystallized from hot petroleum ether and melted sharply at 82° C. (uncorr.). The necessary amount was dissolved in distilled water and neutralized to phenol red. Control experiments showed that it did not interfere with the method of sugar estimation used (Hagedorn and Jensen), even when added to blood in relatively large amounts. The animals used were rabbits which had been starved overnight, and the injection was subcutaneous.

Doses of various sizes were employed and were found to produce different results. The minimal lethal dose was related to the weight of the animal and was 60–70 mg. per kg. body weight. With smaller doses than these, no change was noticed.

With amounts of over 100 mg. per kg., two separate effects were observed. One was on the nervous system and consisted in a general depression of the higher centres. The rabbits first showed fatigue and their chins rested on the floor; this was succeeded by a stage of drowsiness and weakness, in which they lay on their sides; finally, after gasping respirations, they died. The other effect was the well-known one of marked muscular stiffness and rigor, often supervening before death. At death



the animal was usually in complete rigor. With lethal doses of less than 100 mg. per kg. the muscular effect was not often seen, the other alone being marked. The simple glycolytic mechanism of the brain [Ashford and Holmes, 1929] appears to be easily affected by small doses of the acid. In a few cases the heart was suddenly paralysed, but this often occurred after the other symptoms had been present for some time.

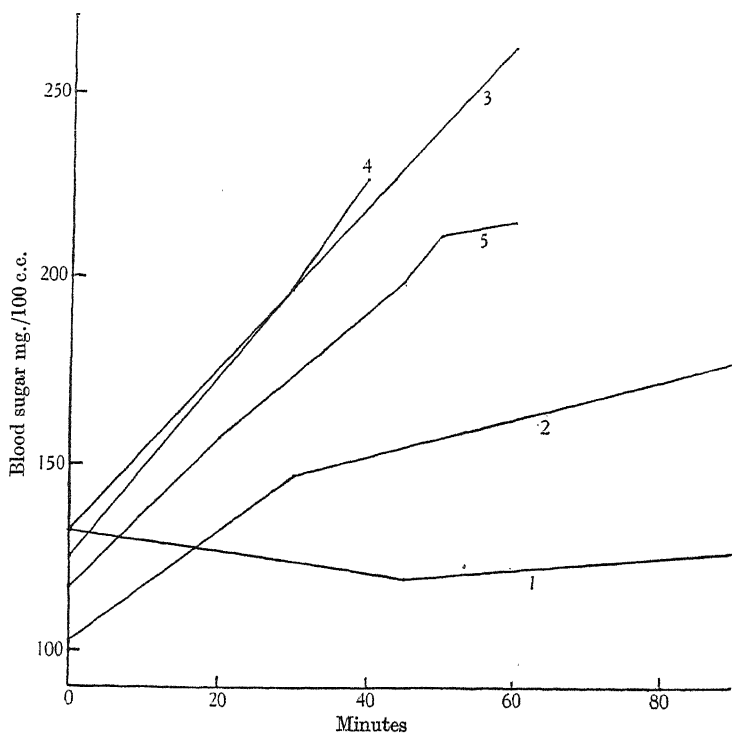


Fig. 1. Influence of iodo-acetic acid on the blood-sugar level. The dosage, in terms of mg. per kg., was respectively: No. 1, 50; 2, 70; 3, 80; 4, 120; 5, 200. The acid was injected at the beginning of the experiment, only No. 1 surviving.

In all cases except two, if the blood sugar rose, the animal died. The same degree of hyperglycemia occurred with all doses above 80 mg. per kg., but with smaller doses it was not usually quite so marked (Fig. 1). It thus seemed that some process was set in motion by lethal doses of iodo-acetate which led, among other things, to hyperglycemia. The possibility at once presented itself that this was due to interference with the centre in the pons which controls carbohydrate metabolism.

[Donhoffer and Macleod, 1932] and was caused, in part at least, by mobilization of liver glycogen, probably by sympathetic impulses. The effects of ergotoxine were therefore tried.

*Ergotoxine*<sup>1</sup>. This was injected intravenously in 5-6 mg. doses as ergotoxine ethanesulphonate, dissolved in dilute acetic acid. The results obtained were not as clear-cut as could have been wished, but showed that the hyperglycæmia was either abolished or considerably reduced. Typical figures are given in protocols 1 to 3. It would thus appear possible that the hyperglycæmia was due to discharge of glycogen following sympathetic stimulation.

*Pituitrin*. Burn [1915, 1923] has shown that pituitrin abolishes adrenaline and anæsthesia hyperglycæmia in rabbits, ergotoxine abolishing the former but not the latter. Pituitrin was therefore tried and was found completely to abolish iodo-acetate hyperglycæmia, as is shown in protocols 4 and 5.

Exp. No.	Min.	Remarks
1	0	1.47 kg. rabbit. 6 mg. ergotoxine injected intravenously.
	25	Blood sugar (cardiac puncture) 0.155 p.c.
	35	200 mg. iodo-acetic acid per kg. injected subcutaneously.
	50	Animal died. Blood sugar 0.139 p.c.
2	0	2.10 kg. rabbit. 5.5 mg. ergotoxine injected intravenously.
	25	200 mg. iodo-acetic acid per kg. injected subcutaneously.
	85	Animal died. Blood sugar 0.113 p.c.
3	0	1.42 kg. rabbit. 6 mg. ergotoxine injected intravenously.
	25	Blood sugar (cardiac puncture) 0.164 p.c.
	30	200 mg. iodo-acetic acid per kg. injected subcutaneously.
	60	Animal died. Blood sugar 0.211 p.c.
4	0	2.14 kg. rabbit. 4 c.c. pituitrin injected subcutaneously into right flank.
	20	200 mg. iodo-acetic acid per kg. injected subcutaneously into left flank.
	23	Blood sugar 0.138 p.c.
	83	Animal died. Blood sugar 0.102 p.c.
5	0	2.01 kg. rabbit. 4 c.c. pituitrin injected subcutaneously into right flank.
	15	200 mg. iodo-acetic acid per kg. injected subcutaneously into left flank.
	19	Blood sugar 0.136 p.c.
	56	Animal died. Blood sugar 0.122 p.c.

Burn [1915] concluded that in rabbits pituitrin acted directly on the liver cells, since it abolished anæsthesia hyperglycæmia which ergotoxine did not do. Since, however, Evans, Tsai and Young [1931] have shown that anæsthesia hyperglycæmia is almost entirely due to the

<sup>1</sup> I am indebted to Prof. J. H. Burn for much useful information with regard to ergotoxine.

liberation of adrenaline, it is fair to assume that pituitrin acts like ergo-toxine in abolishing the action of the sympathetic on the liver. Iodo-acetate has no effect on the breakdown of glycogen in slices of isolated liver as the figures in protocol 6 show. Thus it would seem that the hyperglycæmia has its origin largely in liberation of liver glycogen, which is almost certainly mobilized by the sympathetic.

Exp. No.	Remarks
6	Effect of iodo-acetate on liver glycogenase. 2 g. of liver were suspended in 1 c.c. of water, estimations being done on the whole suspension. Initial glycogen content 1.67 p.c. Glycogen content after 2 hours' incubation at 38° C. 0.48 p.c. Glycogen content after similar incubation with 1 mg. of (neutral) iodo-acetic acid 0.48 p.c.

*Insulin.* Although I had previously found that this was antagonistic to iodo-acetic acid, in no experiment in which they were both given was the minimal lethal dose of the acid altered. Insulin injected in large amounts after iodo-acetic acid had been allowed to act in the animal for a few minutes did not check the hyperglycæmia. The smallest lethal dose of iodo-acetic acid given during deep hypoglycæmia was sufficient to

Exp. No.	Min.	Remarks
7	0	1.91 kg. rabbit. 2 units insulin injected.
	125	Convulsions. Blood sugar 0.048 p.c.
	130	70 mg. iodo-acetic acid per kg. injected.
	160	Died. Blood sugar 0.110 p.c.

raise the blood sugar (protocol 7). A mixed effect was obtained if iodo-acetic acid and insulin were injected simultaneously. In most of these experiments large doses of insulin were used (6 units). With doses of iodo-acetate above 100 mg. per kg. hyperglycæmia developed before death and the insulin was without effect. As the dosage of iodo-acetate was reduced the action of insulin came to the fore, and, although the animal succumbed, no hyperglycæmia, or else hypoglycæmia, was found. It was quite plain which drug was exerting an effect, as, when iodo-acetate was predominant, no insulin convulsions were seen but solely the weakness effect mentioned above, and glucose injections did not save the animals. On the other hand, if they went into typical insulin convulsions, they would usually recover with glucose. The results are shown graphically in Fig. 2. The interesting point about them is that there appears to be here a peripheral hypoglycæmia insulin effect which is not affected by

iodo-acetate in toxic doses. The same thing was found in the perfusion experiments mentioned later.

It had been considered at one stage that iodo-acetate and insulin might react together and mutually inactivate each other, though this was improbable on chemical grounds. This was disproved by mixing small amounts of insulin with large amounts of iodo-acetate and allowing them

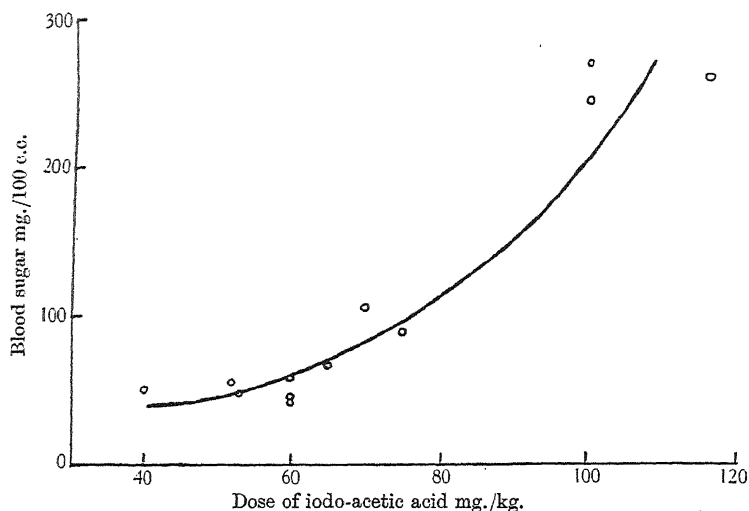


Fig. 2. The influence of the simultaneous injection of insulin (6 units) and iodo-acetic acid (varying amounts) on the blood-sugar level. The blood-sugar values given are those at death or the lowest amounts found.

to stand together for varying lengths of time. On decomposing the iodo-acetate with thiosulphate, the insulin was found to be unimpaired in activity.

#### PERFUSION EXPERIMENTS.

It seemed possible that, in addition to causing hyperglycæmia by mobilizing liver glycogen, iodo-acetate might inhibit the utilization of glucose in the tissues. In order to investigate this, a series of perfusion experiments was undertaken, using rabbits' hindlimbs.

250 c.c. of mixed arterial and venous blood were collected from five to seven rabbits and defibrinated. Usually a small amount of glucose was added. The perfusion was done by gravity at a pressure of 100 mm. Hg. From a reservoir the blood entered a glass spiral, where it was heated, and then traversed the preparation; the outflow passed through a flow

counter and a von Euler and Heymans' oxygenator [1932], being finally returned to the reservoir by a pump worked by hand.

Rabbits of about the same weight were used for the preparation. The one employed was anæsthetized with urethane (1.25 g. per kg. intraperitoneally), the abdominal aorta was cleaned and the lumbar, inferior mesenteric and other arteries in the neighbourhood were ligatured. The aorta was opened and a cannula inserted just below the renal arteries. The animal was then cut transversely at this level and the cannula connected to the apparatus. The preparation was kept warm by a heated plate below and hot-water bottles above and was completely wrapped up in a duster. A thermometer was placed between the preparation and the hot plate and the temperature there was kept at 40° C. As the pump and the glass spiral originally contained approximately 50 c.c. of saline, the total volume of circulating fluid was 300 c.c. The blood was allowed to circulate completely once, and then the first sample was taken and the blood sugar determined. The perfusion was allowed to continue for 1 hour and a further sample was taken. Usually 1-1½ litres of blood flowed through the preparation during that time. It had been hoped to compare the sugar utilization in two separate 1 hour periods in the same preparation, one before, and one after adding iodo-acetate, but it was found that though the sugar utilization was fairly constant from preparation to preparation during the first hour, it always fell away after that time. It was therefore decided to determine the sugar utilization for 1 hour and compare those of different preparations. No difficulty was experienced with pressor substances. During the first few minutes the rate of flow fell owing to vaso-constriction in the preparation, but this never lasted longer than 5 min., and after that a uniform rate was obtained. It was often noticed that iodo-acetate increased the permeability of the capillary walls so that the total volume of blood in the circuit was markedly reduced by the end of 1 hour.

Owing to the expense, not many experiments were performed, but sufficient data have been collected to give a definite answer.

Experiments were first undertaken to compare the sugar utilization of the preparation with and without added iodo-acetate. When the acid was present, it was in a final concentration (allowing for the water content of the preparation) of from 1 : 2000 to 1 : 6000. The results are shown in Table I. It will be seen that in five of the six experiments in which iodo-acetate was present, the utilization of sugar was considerably depressed. Blood glycolysis did not account for more than one-quarter of the sugar disappearing in the control experiments.

TABLE I. Sugar utilization of perfused hindlimbs in the absence and presence of iodo-acetate.

Conc. of iodo-acetic acid (neutral)	Weight of preparation g.	Mg. sugar used per kg. limbs per hour
—	790	111
—	665	110
—	954	68
		Average 96
1 : 6000	777	37
1 : 2100	816	7
1 : 2700	630	0
1 : 2200	700	27
1 : 3500	694	26
(1 : 2200	847	85)*
		Average 19

\* This value has not been included in the average.

Further experiments were undertaken in which insulin was present. In each case 5 units were added to the blood before the experiments started. The results are shown in Table II. As has been found by many

TABLE II. Sugar utilization of perfused hindlimbs in the presence of insulin (5 units), and the absence and presence of iodo-acetate.

Conc. of iodo-acetic acid (neutral)	Weight of preparation g.	Mg. sugar used per kg. limbs per hour
—	850	177
—	760	107
—	754	204
—	770	168
		Average 164
1 : 3600	742	113
1 : 2700	700	131
1 : 2800	718	13
1 : 2800	739	49
1 : 2900	798	113
		Average 84

other workers, the presence of insulin in the control experiments almost doubled the sugar utilization. The results obtained with iodo-acetate in this series were not so clear-cut, but served to show that the utilization of sugar, though lessened, was not nearly so much inhibited as when insulin was absent. This would seem to agree with the results previously referred to, in which lethal doses of iodo-acetate in the intact animal did not prevent insulin hypoglycæmia.

# DISCUSSION.

A perusal of the literature shows that there is little agreement as to the fate of the blood sugar in the peripheral tissues, especially where insulin is concerned. Best, Hoet and Marks [1926] found that in the absence of insulin no glycogen was formed in muscles even if much glucose was present in the blood stream, and this has been confirmed by Choi [1928] and by Markowitz, Mann and Bollman [1929]. On the other hand, Long and Horsfall [1932] found an increase of muscle glycogen under these conditions (though more was laid down if insulin was present), and Cleghorn and Peterson [1932] found the muscle glycogen of decerebrate eviscerate cats to recover after tetanic contraction. Thus, though it would appear that under the action of insulin the increased glucose disappearance is largely seen as glycogen formation, with no insulin present the relative amounts of glucose undergoing oxidation and storage are not definitely known.

From the figures of Best, Dale, Hoet and Marks [1926], it is seen that under the action of insulin, roughly half the sugar used by the muscles was burnt and half stored. The same was found by Cori and Cori [1928]. Although it is not safe to argue from so few figures as those above, it may be not without significance that the degree of inhibition of sugar utilization in Tables I and II is approximately the same, which might be interpreted as meaning that in the absence of insulin oxidation of sugar alone occurs, and that both in the presence and absence of insulin this is the sole mechanism affected by iodo-acetate.

It is generally considered that iodo-acetate inhibits the formation of lactic acid from glucose and glycogen. Recently, however, Brücke [1933] has reported that it will also prevent the formation of glycogen from glucose by yeast. Goldblatt [1933] has also shown that non-lethal doses prevent the accumulation of liver glycogen which normally follows the administration of adrenaline and insulin in young rabbits, and Hikiji [1932] has found that daily injections of small amounts of bromo-acetic acid caused, among other things, a disappearance of muscle glycogen and death in a few days. It is possible that in my experiments iodo-acetate inhibited glycogen formation as well as glucose oxidation. But it does not appear to be permissible to argue from this that lactic acid is a normal intermediate between blood sugar and muscle glycogen, as it has often been shown that lactic acid in the blood stream cannot be converted into muscle glycogen [e.g. Long and Horsfall, 1932; Eggleton and

Evans, 1930]. The only known possible intermediate is dihydroxy-acetone [Kermack, Lambie and Slater, 1929].

It is hoped in future experiments to determine by direct analysis, using a different type of preparation, to what extent muscle glycogen formation is inhibited by iodo-acetate, and if possible what intermediate compounds are involved.

#### SUMMARY.

1. Lethal doses of iodo-acetate always cause a hyperglycæmia, which is often marked.

2. This is largely inhibited by ergotoxine and completely annulled by pituitrin, without altering the toxicity of the acid.

3. Iodo-acetic acid usually reverses or prevents insulin hypoglycæmia. Using appropriate doses, however, it is possible to obtain a hypoglycæmia without affecting the toxic effect of the acid.

4. Iodo-acetic acid markedly inhibits the utilization of sugar by the tissues in the absence of insulin. When insulin is present, this inhibition is not so pronounced.

5. The conclusion is drawn that iodo-acetic acid causes hyperglycæmia by mobilizing liver glycogen and interfering with the disappearance of sugar in the tissues.

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## THE CARBOHYDRATE METABOLISM OF THE KIDNEY.

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THE investigation described in this paper was undertaken to determine the rate at which the kidney used carbohydrate, the relationship of carbohydrate metabolism to urine formation and to total renal metabolism as assessed by the oxygen consumption.

An obvious way of measuring the rate of carbohydrate usage would be to estimate the sugar in blood from the renal artery and vein and multiply the difference by the rate of blood flow. Consideration of the possible rate of carbohydrate metabolism, based on measurement of renal oxygen consumption [Barcroft and co-workers, 1905, 1910; Bainbridge and Evans, 1914; Fee and Hemingway, 1928] and the blood flow, suggested that such a method could not be applied to the kidney because the difference in blood-sugar content would be too small for estimation. Neither would it take account of glycogen which might be stored in or liberated from the organ.

It was therefore decided to use the pump-lung-kidney preparation introduced by one of us [Hemingway, 1931] and to measure the total carbohydrate usage of the whole preparation. The usage due to the kidney can be found by making an allowance for the storage or liberation of glycogen and for the metabolism of the lungs.

### METHODS.

After preliminary experiments had confirmed the impossibility of estimating the difference in sugar content between the arterial and venous blood and the necessity for considering changes in the glycogen content of the perfused organs, the following methods and procedure of experiment were adopted.

(a) *The pump-lung-kidney preparation and procedure of experiment.*

The pump-lung-kidney preparation was set up according to the method previously described [Hemingway, 1931], but an improved pump was introduced into the circuit [Hemingway, 1933]. Other slight modifications of the original method were introduced during the course of the experiments. The apparatus was filled with alcohol when not in use and washed with water and saline immediately before use.

Two dogs were usually used for an experiment, one supplying blood and lungs, the other the kidney. The first dog, weighing 17–18 kg., was anaesthetized and about 450 c.c. of blood removed. This was whipped, twice filtered through organdie muslin [mesh = 0.01 in.] and a measured volume introduced into the apparatus.

Meanwhile a cannula was introduced into the pulmonary artery, the lungs were removed from the thorax and after weighing, perfusion was commenced. The lungs were accommodated on a large Büchner funnel and positively ventilated by a Schuster pump instead of being kept in a closed vessel, as originally described, because it was necessary during an experiment to remove samples of lung for glycogen estimation.

The second dog was anaesthetized while the lung preparation was being set up and the left kidney was removed and perfused as soon as the pulmonary flow was established. Simultaneously a slice of the right kidney was removed for glycogen estimation. When the blood flow through the kidney was established, and constant (usually within 2 or 3 min. of the attachment of the kidney to the apparatus) a portion of the lung was tied off and removed for estimation. Simultaneously, blood was taken for blood-sugar determination.

The output of the pump was constant in any one experiment and the fixed perfusion pressure to the kidney was regulated by passing some of the blood through a shunt directly to the lungs. This ensured that the flow through the lungs was constant and independent of that through the kidney.

The perfusion was allowed to continue for 40–50 min., and, at the end of the period, samples of blood, lung, and kidney were taken for estimation. The samples of lung and kidney were the counterparts of those taken for the first estimations. That the glycogen content of corresponding portions of the organs is similar is shown by Table I, and confirmation is given by Irving [1928 b] for the kidney.

TABLE I. Glycogen content of right and left kidneys and lungs.

Exp.		Glycogen mg./100 g.
18	Right lung	177
	Left lung	170
24	Right kidney	68
	Left kidney	70

The perfusion apparatus was then drained and the volume of remaining blood measured; the lungs and the kidney were weighed. The difference between the initial and final amounts of carbohydrate in the system when calculated gave the quantity of this material used by the kidney and lungs. Samples of urine were tested from time to time for reducing substance but none was found.

In other experiments the sugar used by the lungs alone was measured, and in these an exactly similar technique was followed except that the kidney was not added to the circuit.

(b) *Estimation of glycogen.*

As a result of experience a modification of the Pflüger method of glycogen estimation based on that described by Kerly [1930] was adopted. A fragment of tissue judged by eye to be about 4 g. was dropped into a previously weighed tube containing 5 c.c. of 60 p.c. KOH. The exact weight of the tissue was immediately determined and the tube placed in a boiling water bath. After about  $2\frac{1}{2}$  hours the alkaline digest of the tissue was cooled and neutralized by 60 p.c. trichloroacetic acid. The neutralized samples were made up to 25 c.c. and filtered. 20 c.c. of the filtrate were added to 80 c.c. of alcohol and allowed to stand in the ice chest overnight. The glycogen was separated by centrifuging and taken up in 15 c.c. of  $N H_2SO_4$ , and heated on a water bath for 2 hours. After hydrolysis the acid was neutralized by an accurately measured quantity of 60 p.c. KOH and made up to 25 c.c. Finally, samples were analysed for glucose by the Somogyi modification of the Shaffer-Hartmann method [Peters and van Slyke, 1932] and the glycogen values expressed in terms of reducing sugar in the hydrolysate.

(c) *Estimation of blood sugar.*

Blood-sugar determinations were made by the Somogyi modification of the Shaffer-Hartmann method on protein-free blood filtrate prepared after the method of van Slyke and Hawkins [1928]. Not only was it desirable to use the same method for the determination of reducing

substances in blood and in glycogen hydrolysates, but our experience indicated that this method was in many ways the most convenient in practice.

#### RESULTS.

Separate consideration has been given to (a) the "true" metabolism of the lungs, making allowance for storage or release of glycogen, (b) the rate of disappearance of sugar from circulating blood and (c) the usage of sugar by the kidney.

##### (a) *The metabolism of the lungs.*

Previous investigations have been made on the metabolism of perfused lungs by Evans and Starling [1913], Patterson and Starling [1913] and Cruickshank and Startup [1930]. Patterson and Starling examined the sugar metabolism and concluded that the average rate of usage was 1.3–1.5 mg./hour/g. of heart muscle. These earlier investigations did not take into reckoning that some of the sugar disappearing from the blood might not be oxidized but, on the contrary, synthesized to glycogen. Cruickshank and Startup reopened the question with this point in view, and by making simultaneous measurements of oxygen consumption, respiratory quotient, and rate of sugar disappearance from the circulating blood, were able to measure with greater certainty than before the amount of sugar actually used and that stored or liberated. They gave 0.7–0.8 mg./hour/g. of heart muscle as the rate of usage. These results, as well as those of Patterson and Starling, are expressed in terms of heart muscle weight rather than in lung weight, because in their experiments the lungs tended to become cedematous, thus making the correct determination of lung weight difficult.

During the present experiments it was thought advisable, for two reasons, to redetermine the rate of lung metabolism, (a) glycogen changes were being investigated by a direct method instead of the indirect methods of previous investigators and (b) the method of pulmonary perfusion employed was dissimilar to those previously used.

The results of a typical experiment in which the rate of lung metabolism was measured are given in the accompanying protocol (see p. 373).

The diminution which occurred in the amount of circulating blood was attributed after further experiment to evaporation from the outer surface of the lungs. Such loss was minimized by covering the Büchner funnel with a damp cloth, but even so it was always sufficient to necessitate careful measurement of the residual blood at the end of an experiment.

*Protocol of Exp. 24.*

	Amount	Carbohydrate concentration	Total carbohydrate mg.
	<i>Beginning of period.</i>		
Lungs	94 g.	154 mg./100 g.	145
Blood	461.5 c.c.	112 mg./100 c.c.	<u>516</u>
			661
	<i>After 46.5 min. perfusion.</i>		
Lungs	100 g.	182 mg./100 g.	182
Blood	448 c.c.	94 mg./100 c.c.	<u>421</u>
			603

58 mg. of carbohydrate used by lung (average weight 97 g.) in 46.5 min. Rate of usage = 0.013 mg./g./min.

Three experiments were performed and the average of the three determinations gave the carbohydrate metabolism of the lungs as equivalent to 0.014 mg./g./min. The findings in the individual experiments are given in Table II.

TABLE II. Glucose usage of perfused isolated lungs.

Exp.	Wt. of lungs g.	Glucose usage mg./g./min.
3	100	0.014
23	126	0.015
24	97	0.013

This rate of metabolism is in good agreement with that found by Cruickshank and Startup, whose experiments, taking the lung weight as 87 p.c. of the heart weight [Evans and Starling, 1913], show a lung usage of 0.013–0.015 mg./g./min. The results are expressed in terms of lung weight and not heart weight, because the lungs were weighed at the beginning and the end of the experiment and little or no cedema was found. The changes in lung weight occurring in three successive experiments are given in Table III and are typical of the whole series.

TABLE III. Weight of lungs before and after perfusion.

Exp.	Wt. before perfusion g.	Wt. after perfusion g.
24	94	100
25	130	126
26	166	173

*(b) The disappearance of carbohydrate from circulating blood.*

Cruickshank and Startup [1932] investigated the rate at which sugar disappeared from blood which was mechanically circulated and oxygenated, and in a summary of their results stated that in 3 hours the

loss of sugar was 42.5 p.c. of the original total. On this account, it was considered whether a correction should not be applied to our results, making an allowance for sugar disappearing from the blood but not metabolized by the perfused tissues.

To obtain data for a correcting factor, similar experiments to those performed by Cruickshank and Startup were performed, but different samples of blood showed changes both greater and smaller in amount than their results indicated. Also, it was debatable whether it would be reasonable to make any comparison of changes occurring in blood circulating through tissues and in blood mechanically circulated and oxygenated, knowing that there are differences between such bloods, *e.g.* the difference in vaso-constrictor properties. Because of this, and our inability to obtain a constant figure, no correction has been made for carbohydrate disappearing from the blood but not metabolized by the tissues.

(c) *The glucose usage of the kidney.*

The results obtained for the kidney require little explanation. The total usage of the lungs and kidney was determined over a period and then an amount corresponding to the weight of the lungs was subtracted on the assumption that the rate of usage was 0.014 mg./g./min. Eight experiments were performed, using the described technique, and in Table IV are given the results of five, none of which showed any technical fault to careful scrutiny. In these experiments the rate of sugar usage varied between 0.014 and 0.099 mg./g./min. The figures are of the same order as those given by Irving [1928 *a*] for the glucose usage of minced rabbit kidney cortex which varied between 0.018 and 0.058 mg./g./min.

TABLE IV. Summary of experiments in which the glucose usage of kidney has been measured.

Exp.	Wt. of kidney g.	Glucose usage mg./g./min.	Urine c.c./min. (average)	Blood flow		Perfusion pressure mm. Hg
				c.c./min. (max.)	c.c./g./min.	
20	48.3	0.041	0.30	243	5.0	120
25	24.8	0.014	0.31	91	3.7	116
27	58.9	0.036	0.28	—	—	120
28	42.8	0.099	0.52	217	5.0	120
29	36.6	0.046	1.04	186	5.1	140

### DISCUSSION.

The results seem to invite the discussion of two main points, (a) the relationship of the rate of glucose usage to renal activity and (b) the ratio of carbohydrate metabolism to total metabolism in the kidney. It is not

easy to make a definite statement regarding the first of these points because the readings have been taken over a period during which renal activity might be varying. Nevertheless, in general the experiments in which a high glucose usage has been recorded were those in which there was a large blood and/or urine flow. Indeed if the weight of the kidney be taken into consideration the rate of glucose usage appeared to follow the rate of blood flow. This result is natural enough if the tubule cells are regarded, as they must be, as doing work in transferring materials and water from blood to tubule lumen and *vice versa*, but on the other hand, observations of the rapid flows in the kidney treated with cyanide or asphyxiated show that little reliance can be placed upon the rates of urine and blood flow as true indicators of renal activity.

The second point leads to a more definite conclusion. It will be realized that if the oxygen used by the kidney [Fee and Hemingway, 1928] is employed solely in the oxidation of carbohydrate, then the glucose usage should fall within the limits of 0.039 and 0.26 mg./g./min. The figures recorded here are much below these rates, but the ratios for maximal and minimal values, between the measured and the highest possible glucose usage, as calculated from the oxygen consumption, are in remarkable agreement as is shown in Table V.

TABLE V. Relationship between measured rates of glucose usage and those calculated from the oxygen consumption [Fee and Hemingway, 1928].

	Rate of glucose usage mg./g./min.		Ratio <u>measured</u> calculated
	Measured	Calculated from oxygen usage	
Minimal	0.014	0.039	0.36
Maximal	0.099	0.260	0.38

This close agreement, which suggests that about one-third of the oxygen used is employed for carbohydrate metabolism, assuming that the carbohydrate which disappears is completely oxidized, may be purely fortuitous and the exact differentiation of the usage of oxygen between carbohydrate and other substances can only be found in an individual experiment by determining the R.Q. of the kidney. The difference between the two sets of figures measured and calculated is however so great (and is actually increased if the whole of the carbohydrate which disappears is not completely oxidized) that it is certain that the whole of the kidney metabolism is not due to oxidation of carbohydrate.

The early experiments of Bainbridge and Evans [1914] on the

R.Q. of the kidney in the heart-lung-kidney preparation show a quotient of less than unity and an average of 0.71, and suggest that the proportion of carbohydrate metabolism to the whole is very small.

#### SUMMARY.

1. A method of measuring the carbohydrate metabolism of a perfused organ is described.
2. The carbohydrate usage of the perfused isolated lung, estimated as glucose, is 0.014 mg./g./min.
3. The carbohydrate usage of the perfused kidney varies between 0.014 and 0.099 mg./g./min.
4. About one-third of the oxygen used by the kidney is employed in the metabolism of carbohydrate.

The expenses of this research were defrayed in part by a grant to one of us (A. H.) from the Government Grants Committee of the Royal Society to whom our thanks are due.

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## ON THE OCCURRENCE OF TWO KINDS OF HÆMOGLOBIN IN NORMAL HUMAN BLOOD.

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IN the fifth chapter of Barcroft's book on hæmoglobin he discusses its specificity, and suggests the possibility of more than one hæmoglobin in the same blood, the differences being located in the globin part of the molecule. The curious change in "span" (*i.e.* the distance between the bands for oxy- and CO-hæmoglobin) in the blood of the same rabbit makes Barcroft put the question, "Why and how should the globin of a rabbit's hæmoglobin alter on hæmorrhage?"

In limiting the problem to the unity and variability of hæmoglobin in one kind of blood, especially in human blood, we find that most methods of research on the specificity of hæmoglobin are not sensitive enough to detect the presence of, say, 10 p.c. of a modified hæmoglobin in 100 p.c. total. Only the estimation of the so-called alkali resistance, which gives enormous differences in blood of different species, might be sensitive enough. This paper describes the photo-electric measurement of the rate of denaturation of human hæmoglobin at *pH* about 12, as a means of differentiation between more than one kind of pigment in the same blood.

### METHOD.

Von Krüger [1888, 1925, 1927], who first studied the rate of hæmoglobin denaturation in alkaline solutions ("Zersetzungszeit"), added 1/5 vol. *N*/4 NaOH to the solution of hæmoglobin and measured the time from this moment to the visual disappearance of the typical absorption bands. In this way he found the very large differences in "Zersetzungszeit" in the hæmoglobin of mammalian species, for instance for human blood 1 min., for rabbit's blood 30 min., for horse blood 80 min., for ox blood 24 hours. A more exact study was made by Haurowitz

[1929], who measured the rate of extinction in the region of the  $\beta$  bands (538–544) and also in the  $\alpha$  region (572–580) by means of the spectrophotometer.

For production of the alkaline reaction he used the same process as von Krüger, viz. the addition of  $1/5$  vol.  $N/4$  NaOH.

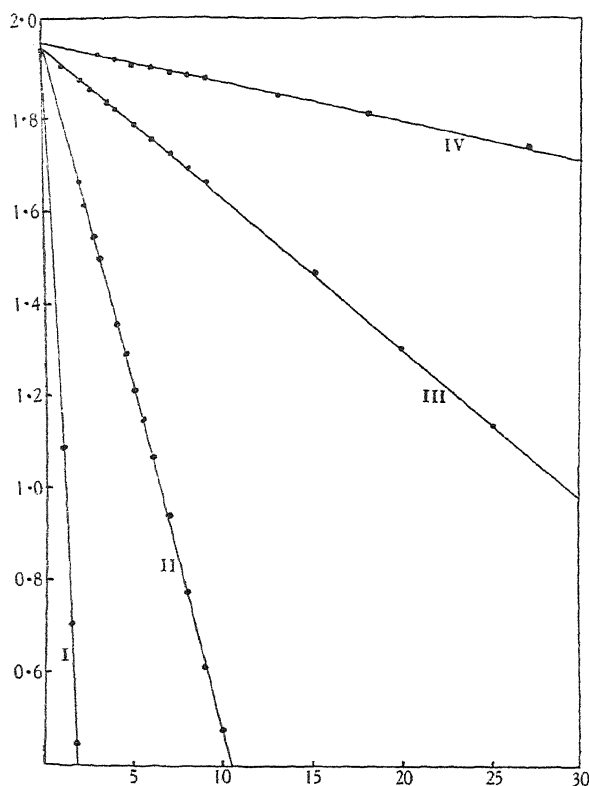


Fig. 1. Effect of pH on rate of alkali denaturation at 19.6° C. Vertical axis: log percentage unchanged haemoglobin in total mixture; horizontal axis: time in minutes. (I) pH 12.12; (II) pH 11.89; (III) pH 11.66; (IV) 11.55.

The sensitivity of this method is very much dependent on the investigator's experience, and on the time which may be taken for each determination. In our opinion it will be difficult to estimate a decrease in extinction, smaller than that corresponding to a 2 p.c. change of oxyhaemoglobin to globin haemochromogen. For our purpose we have tried to combine a good sensitivity with the exclusion of the subjective

factor, by using a photo-electric arrangement, and by keeping the conditions which govern the speed of reaction sufficiently constant.

The two factors on which the rate of alkali denaturation of a certain type of hæmoglobin is dependent are (a) the concentration of OH ions, and (b) the temperature.

Fig. 1 shows the influence of  $pH$  on the rate of denaturation at constant temperature. The hæmoglobin was obtained from a human blood, which did not contain more than 5 p.c. of the resistant form of hæmoglobin (to be described later). The ratio's log conc.  $HbO_2$  : time are straight lines for about 80 p.c. completion of the reaction.

The solution of oxyhæmoglobin was made, by diluting oxalate blood with 0.15  $N$   $Na_2HPO_4$  solution, containing 1 p.c. of saponin, the final concentration of hæmoglobin being 1.36 p.c. This solution was centrifuged till it was quite clear and put into the absorption vessel of 1 cm. transverse diameter. The alkali denaturation was started by injection of 1 c.c.  $NaOH$  of various concentrations, by means of a precision syringe.

A special set of experiments showed that the described procedure enabled us to place the oxyhæmoglobin quickly in a medium of known and sufficiently constant  $pH$ ;  $pH$  in each experiment was determined by the bubbling hydrogen electrode. Great care was taken to keep change of temperature in the reaction vessel within  $0.1^\circ C$ .

The temperature coefficient of protein denaturation being in many cases very large [Pauli, 1933], it is not surprising that a slight change in temperature has a definite effect on the rate of formation of globin hæmochromogen. In our experiments the temperature did not change over more than  $0.1^\circ C$ . in each measurement, although differences of temperature between separate experiments may be larger.

#### THE COLORIMETRIC ESTIMATION OF THE RATE OF CHANGE OF OXY-HÆMOGLOBIN TO ALKALINE GLOBIN HÆMOCHROMOGEN.

We used two methods, a bicolorimetric estimation without standard solutions and a photo-electric determination with calibration by means of known mixtures of  $HbO_2$  and globin hæmochromogen.

The second method being the more exact one, it was solely used later on, although the bicolorimetric procedure too can give the rate of denaturation with sufficient sensitivity and may be briefly described for the use of those who wish to make analogous measurements.

Human, hæmolyzed blood, containing 2.78 mg. Hb per c.c. was saturated with pure  $CO$ , made very clear by centrifugalization and divided in  $3 \times 5$  c.c. in test-tubes *A*, *B* and *C*. Carboxyhæmoglobin is used, because its change on alkaline denaturation gives the most pronounced change in colour; the same observation has been made for the bicolorimetric determination of methæmoglobin [Clark, 1933]. To *A* is given 5 c.c. buffer solution so that the reaction is fixed at  $pH$  12.00 at  $18^\circ C$ .; the tube is then placed at  $37^\circ C$ . for 15 min.,

the oxyhæmoglobin is thereby completely converted into the darker denaturation product. After addition of 5 c.c. distilled water to *B*, 5 c.c. of *A* and of *B* are filled in the lower and upper right cups of the colorimeter. *C* is placed in a 25° C. bath, together with a tube, containing 5 c.c. of the alkaline buffer solution. The contents of these two tubes are then quickly mixed and poured into the left colorimeter cup, fixed at 4 cm. distance and having a constant temperature circulating water jacket. From this moment the stopwatch is started, and colours are matched by making suitable optical mixtures of *A* and *B*, the total depths of liquid at both sides remaining 4 cm.

The best light for sufficient sensitivity was obtained by illuminating a piece of paper, brilliantly painted in the complementary colour of carboxyhæmoglobin, by a powerful Liesegang globoscope and sending the reflected light directly through the colorimeter cup; the light was turned off between measurements. The bicolorimeter readings are directly proportional to the amount of oxyhæmoglobin in total pigment; the method will indicate a change of about 3 p.c. of conversion. The results obtained were similar to those given by the photo-electric method which will now be described.

A method, suitable for our purpose, must be capable of distinguishing quantitatively between, say, a mixture of 95 p.c. globin hæmochromogen and 5 p.c. oxyhæmoglobin and a mixture of 94.5 and 5.5 p.c. respectively. We were not able to obtain a suitable monochromatic light of sufficient strength, but use a  $\text{CuSO}_4$  filter, which cuts off the extreme red, which is not absorbed by globin hæmochromogen and to which the selenium cell is rather sensitive.

A 1000 c.p. Pointolite lamp is connected in series with an extra resistance and galvanometer; during the measurement an assistant regulates the current continuously at exactly 3 amp., which ensures sufficient steadiness of illumination and discards with the necessity of a differential method. An 8.0 lens concentrates the light on the surface of a selenium-oxyde "Sperrschicht" photo-electric cell, after it has passed through a  $\text{CuSO}_4$  filter and the hæmoglobin solution (20 mm. of a 1.2 p.c.  $\text{CuSO}_4$  solution). The selenium cell is connected in series with an E.M.F. of 300 mv. and a 10,000 ohm fixed resistance; the drop in potential over this resistance is measured by a Cambridge electrostatic valve potentiometer.

The sensitivity of this arrangement is seen in Fig. 2; for our purpose the most important change is that from 90 p.c. globin hæmochromogen and 10 p.c. oxyhæmoglobin to 100 p.c. of globin hæmochromogen. This gives for a 1.0 p.c. solution of human oxyhæmoglobin a drop of 28 mv., with an accuracy of at least 1 mv.; for the change from 80 to 100 p.c. globin hæmochromogen the drop is 61 mv.

If the concentration of hæmoglobin is doubled, the drop for the last 10 p.c. of the native pigment is only 20 mv., because the total absorption of light is too large. For this reason the best way is always to start with the same concentration of oxyhæmoglobin, and to use the calibration

curve for this concentration. If the percentage of oxyhæmoglobin differs more than 10 p.c. from this, a corresponding, separate calibration curve is wanted. One experiment will now be described.

3 c.c. oxalate blood from the finger, containing 16.8 g. of hæmoglobin per 100 c.c. were delivered in a 50 c.c. volumetric flask, containing 0.15 *N*  $\text{Na}_2\text{HPO}_4$  and 1 p.c. of saponin, so that the total volume was 50 c.c. After 5 min. this solution was put into a very efficient small centrifuge, so that a very clear liquid was obtained. The 1000 c.p. Pointolite lamp had already been alight for 10 min. at 3.0 amp.; the drop in potential over the 10,000 ohm fixed resistance was 835 mv. with an accuracy of 0.5 mv. when the absorption cuvette was filled with water; the same drop with darkened selenium cell was 117 mv.

10 c.c. of the prepared solution were delivered in the 10 mm. absorption cuvette; the E.M.F. over 10,000 ohm was then 710 mv. Temperature was regulated by circulation of water around the circular, metal side-wall of the absorption cuvette; in this experiment the temperature in the cuvette during the reaction was  $18 \pm 0.04^\circ \text{C}$ . 1 c.c. of 0.9 *N* NaOH solution was injected into the filled cuvette by a calibrated tuberculin syringe, and well mixed by repeated suction and pressure. The stopwatch was started at this moment, and the decreasing E.M.F. over 10,000 ohm was determined every 30 sec. The potentiometer used was the new Cambridge electrometer valve potentiometer. Afterwards the reaction of the solution in the cuvette was determined by the bubbling hydrogen electrode at  $p\text{H} = 11.86$ .

#### *Calibration.*

After completion of denaturation in the cuvette the reaction was adjusted to about  $p\text{H}$  8 by addition of 0.5 c.c. normal acetic acid. This may cause a precipitation of the denatured hæmoglobin, which re-dissolves at once when it is well mixed. A too gradual neutralization, for instance by  $\text{CO}_2$  bubbles, may cause a true partial reversion of denaturation [Anson and Mirsky, 1931]. If one accounts for the dilution, caused by addition of 0.5 c.c. of acetic acid to 11 c.c. of globin hæmochromogen, the absorption in the cuvette has not changed by the partial neutralization.

Mixtures of the denaturated solution at  $p\text{H}$  8 with the original solution of oxyhæmoglobin (diluted accordingly from 10 to 11.5) are now prepared by adjusting the amount of denatured hæmoglobin at  $p\text{H}$  8 to a volume of exactly 10 c.c., and by exchanging 1 c.c. of it for 1 c.c. oxyhæmoglobin solution, and so on. The calibration curve, giving the relation of the percentage of unchanged oxyhæmoglobin in total pigment to drop in E.M.F.

over the 10,000 ohm resistance is seen in Fig. 2. The calculation of the rate of denaturation in percentages of unchanged oxyhæmoglobin from the observed drop in millivolts is made by this corresponding calibration curve.

### Results.

The constant result of the examination of human oxyhæmoglobin of normal persons, aged 7-50 years, is the fact that the reaction first proceeds rapidly, then more slowly. If we make the assumption that there

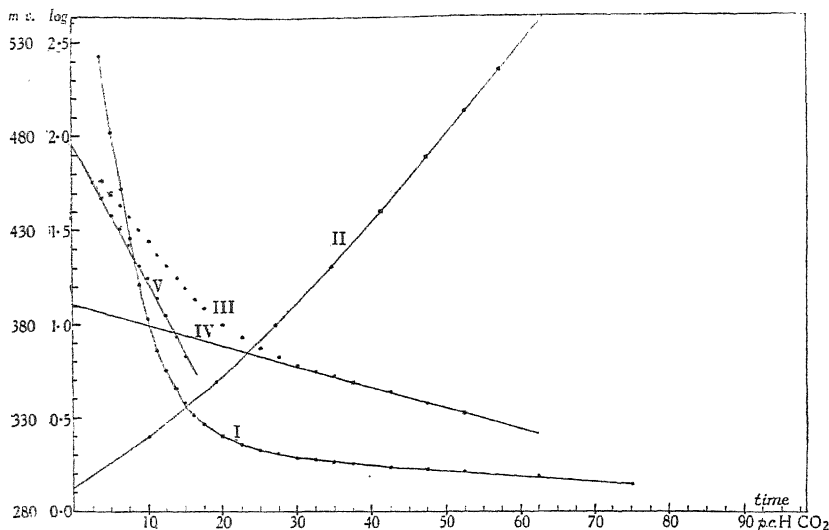


Fig. 2. Rate of alkali denaturation of the hæmoglobin of R. B., in 1.0 p.c. solution. Description in text, p. 384. I. Observed drop in millivolts. II. Calibration curve. III. Log percentage of unchanged  $C_1 + C_2$ . IV. Extrapolation of  $C_2$ . V. Log  $C_1$ . Vertical axis, millivolts and log percentage unchanged hæmoglobin. Horizontal axis, time in minutes and percentage unchanged hæmoglobin (for II).

is no appreciable change in pH value, or other secondary reactions affecting the velocity constant during the course of the main reaction, the diminution in the reaction velocity may be attributed to the existence of a resistant form of hæmoglobin. Control experiments show that the change in pH is negligible. In an experiment similar to the one described, the pH was determined every minute by the bubbling hydrogen electrode; 1 min. after the addition of 0.9 N NaOH 1 c.c. pH was 11.83, 1 min. later 11.82, 2 min. later 11.82, 4 min. later 11.81, 6 min. later 11.80, 10 min. later 11.79, 15 min. later 11.79, 20 min. later 11.79.

TABLE I.

$t$ min.	mv.	Percentage unchanged $C_1 + C_2$	Log unchanged $C_1 + C_2$	Extrapol. log $C_2$	Extrapol. $C_2$	By subtraction $C_1$	Log $C_1$	Percentage unchanged hæmoglobin	Calibration mv.
0	710.0	100.0	2.000	1.108	12.80	87.20	1.941	0	292
1.0	562.0	68.7	1.836	1.080	12.00	56.70	1.754	10.0	320
1.5	522.0	58.6	1.768	1.068	11.70	46.90	1.671	19.0	349
2.0	482.0	49.3	1.693	1.052	11.30	38.00	1.580	27.1	380
2.5	452.0	43.1	1.634	1.040	10.96	32.14	1.507	34.4	411
3.0	426.0	37.6	1.575	1.027	10.64	27.00	1.431	40.9	441
3.5	401.0	32.0	1.505	1.012	10.52	21.50	1.312	46.9	470
4.0	383.0	27.8	1.444	1.000	10.00	17.80	1.250	52.2	494
4.5	366.0	23.4	1.369	0.988	9.73	13.67	1.137	57.0	516
5.0	355.0	20.5	1.312	0.972	9.38	11.12	1.046		
5.5	345.0	17.7	1.248	0.960	9.12	8.58	0.934		
6.0	338.0	15.6	1.193	0.947	8.85	6.75	0.830		
6.5	331.0	13.5	1.130	0.932	8.55	4.95	0.695		
7.0	327.0	12.2	1.086	0.920	8.32	3.88	0.589		
8.0	320.0	10.0	1.000	0.891	7.78	2.22			
9.0	316.0	8.6	0.934	0.862	7.28	1.32			
10.0	313.0	7.55	0.878	0.838	6.89				
11.0	311.0	6.8	0.832	0.810					
12.0	309.0	6.1	0.785	0.785					
13.0	307.5	5.7	0.756						
14.0	306.5	5.35	0.728						
15.0	305.5	5.0	0.699						
17.0	304.0	4.4	0.644						
19.0	302.5	3.85	0.586						
21.0	301.2	3.35	0.525						
25.0	299.0	2.3	0.362						
30.0	296.0								
40.0	292.5								
50.0	292.0								

In Table I and Fig. 2 the result of the experiment described is analysed from this point of view. Calculations are made on the assumption that the hæmoglobin was composed of two kinds, with different rates of denaturation, the concentration of the type with the largest resistance against alkali denaturation being  $C_2$ , and of the other form  $C_1$ . Experimentally found is the amount of  $C_1 + C_2$  which is not denatured after a given time; the logarithms of these concentrations, plotted against time, show that from 12 min. after the beginning of the reaction the points fall on a straight line. This is interpreted as the monomolecular denaturation reaction of  $C_2$ , and extrapolation of  $\log C_2$  to  $t=0$  gives 12.8 p.c. as the initial concentration of  $C_2$ . Subtraction of the extrapolated corresponding values of  $C_2$  from  $C_1 + C_2$  gives the true rate of denaturation of  $C_1$ , which also seems to proceed as a monomolecular reaction.

In Fig. 3 a number of determinations on the hæmoglobin of R. B. are collected;  $pH$  and temperature during each experiment were constant but varied slightly in the separate examinations, as it is recorded in the legend. The denaturation velocity of  $C_1$  is not designed here, but it was represented by a straight line in each case. Our interpretation is that the alkali denaturation of human oxyhæmoglobin is going on as two monomolecular reactions with different velocity constants. The values of these constants can of course be calculated, but the main object of this paper is the demonstration of the existence of two kinds of hæmoglobin in adult human blood. It is seen, that in the blood of R. B. the log of the concentration of the resistant form is found between 0.93 and 1.3, giving the amounts of resistant hæmoglobin as 8.5 to 20 p.c. of the total amount.

In the blood of other persons the results were quite similar, with a tendency in children to show higher values for the resistant form. The examination of the blood of the umbilical vein in new-born children is described by lines A and B in Fig. 3. In confirmation of former researches [von Krüger, 1927; Bischof, 1926; Haurowitz, 1929] the amount of resistant hæmoglobin in neonati is about 80 p.c. (similar determination in six cases).

Hæmoglobin of normal rabbit's blood, and the blood of horse, pig and ox, examined in the same way, but at  $pH$  12.50– $pH$  13 has always given one straight line, indicating the presence of only one kind of hæmoglobin. How this may be altered in experimental anæmia is a subject under research now.

Checks have been made with known mixtures of hæmoglobin of adults and of new-born children. A typical example is given in Fig. 4. Here,



line I gives the rate of denaturation of adult hæmoglobin ( $pH$  12.20,  $t$  17.7° C.), line II the rate of denaturation of new-born's hæmoglobin at

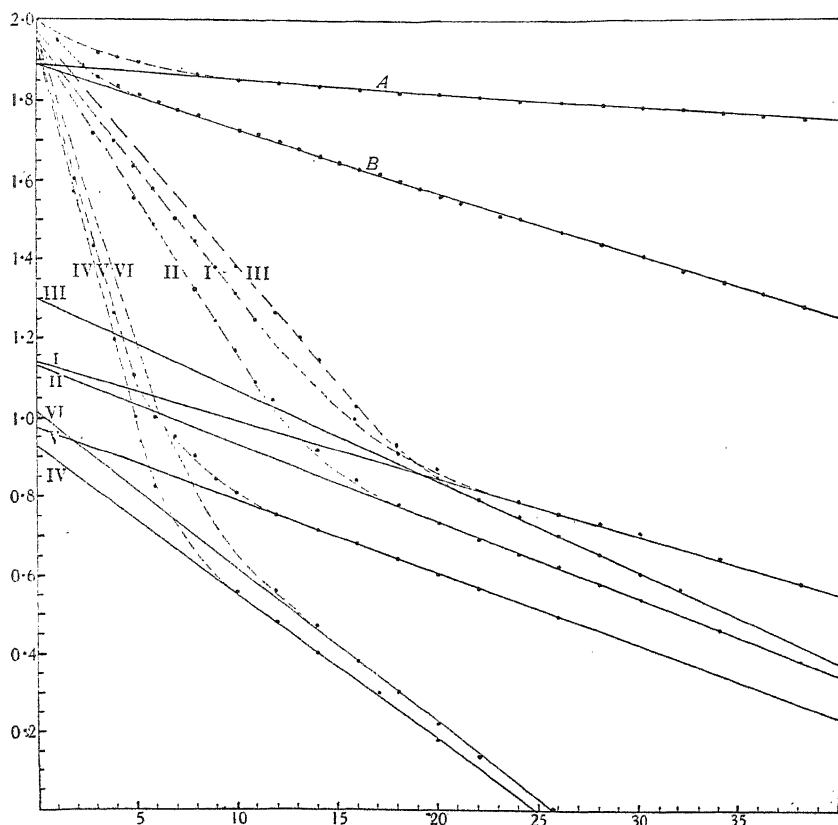


Fig. 3. Rate of alkali denaturation of the hæmoglobin of R. B., in 1.0 p.c. solution:

I.	Sept. 4, 1933.	$pH=11.80$ , $t=18.2^{\circ}$ C.
II.	" 5, "	$pH=11.80$ , $t=18.0^{\circ}$ C.
III.	" 10, "	$pH=11.80$ , $t=17.2^{\circ}$ C.
IV.	" 14, "	$pH=11.86$ , $t=18.6^{\circ}$ C.
V.	" 21, "	$pH=11.84$ , $t=18.5^{\circ}$ C.
VI.	" 28, "	$pH=11.84$ , $t=18.8^{\circ}$ C.

A. Hæmoglobin in 1.0 p.c. solution of new-born child  $pH=12.00$ ,  $t=19^{\circ}$  C.

B. Hæmoglobin in 1.0 p.c. solution of new-born child  $pH=13.00$ ,  $t=19^{\circ}$  C.

Vertical axis, log percentage unchanged hæmoglobin; horizontal axis, time in minutes.

the same  $pH$  and temperature, and line III the denaturation velocity of a mixture of 80 p.c. of I and 20 p.c. of II. It is seen that I indicates the presence of 7.1 p.c. of the resistant type, II gives 93 p.c. of this kind of

hæmoglobin and from III 19.2 p.c. is found; calculation from I and II gives 18.6 p.c. Further, it is seen that one cannot say that the resistant type of adult human hæmoglobin and the new-born's human hæmoglobin are identical. It must be the object of a further study to compare the velocity constants of denaturation of various human hæmoglobins of normal and pathological cases more carefully. In this paper the demonstration of two kinds of hæmoglobin in human blood was intended.

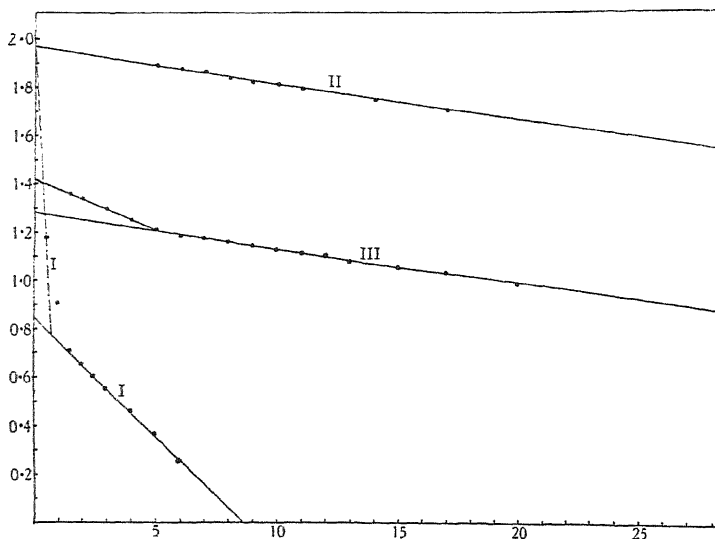


Fig. 4. Denaturation rates of adult hæmoglobin (I), new-born's hæmoglobin (II), and of a mixture of 80 p.c. I and 20 p.c. II. Description in text (p. 385). Vertical axis, log percentage unchanged hæmoglobin in total mixture; horizontal axis, time in minutes.

#### DISCUSSION.

It appears to be generally accepted that, if an eventual difference in various hæmoglobins is present, this specificity must be located in the globin part of the molecule. Evidence for this supposition is growing continuously; apart from the physico-chemical methods of difference in absorption spectra, of crystal form, of solubility and of differences in isoelectric zone and rate of alkaline denaturation, we have the direct chemical analyses of globin. The researches of Schenck [1930] demonstrate a constant difference in the amount of certain amino-acids in the globins of various species. In human blood, especially, the arginine content of globin is constantly higher in the blood of new-born children than

in adult blood. In hypochrome cases of anæmia with normal regeneration this juvenile type seems also to be present; in pernicious anæmia it is missing.

Schenck also shows that the chemical differences of human globins correspond to their different resistances to hydrolysis by pepsin HCl.

Unsolved is the question whether human hæmoglobin is changing from one form to the other, or whether there are two, originally different forms (*e.g.* the resistant "foetal" type and the adult form). The sharp distinction which can be found in the rates of denaturation suggests the existence of two independent kinds, not connected by transitional types.

### SUMMARY.

The rate of alkali denaturation of hæmoglobin, studied by means of a sensitive photo-electric arrangement, was determined in human hæmoglobin. It is shown that, in normal blood, alkaline denaturation is proceeding as two monomolecular reactions with greatly differing velocity constants. This indicates the presence of two kinds of hæmoglobin in human blood.

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## SOME FORMS OF APPARATUS FOR THE EQUILIBRATION OF BLOOD.

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(*Received November 3, 1933.*)

DILL in the appendix to *Blood* by L. J. Henderson [1928] described an excellent technique for the equilibration of blood and its transfer to the van Slyke manometric apparatus. He says, "We propose to describe a technique which we have used when as much as 135 c.c. of blood... were available." The methods described below make no profession of improving upon the accuracy of that technique; their aim has been, while yielding as little as possible in the matter of accuracy, to use very much less blood. The van Slyke manometric apparatus analyses 1 c.c. or even 0.2 c.c. of blood with great accuracy, but the whole process of equilibration, involving the use of large dead spaces, etc., demands, for the determination of dissociation curves, quantities of blood of a much higher order—quantities that are often quite unattainable.

The apparatus used requires little description; several forms have been employed, according to the amount of blood available.

### I. FOR DUPLICATE ANALYSES OF 1 C.C. SAMPLES.

The apparatus is shown in Fig. 1. The main saturating vessel *A* is of about 350 c.c. capacity. It is of about 25 cm. length from *M* to *G*. The stopper is of glass and is of special design. It consists of three concentric tubes. The outermost is the stopper proper. The cavity between this and the middle tube communicates with the air by a three-way tap *F*. This tap is for the injection of the blood. The middle tube is produced about  $1\frac{1}{2}$ –2 cm. (to *D*) further into the saturator than the stopper. It ends in a flange, otherwise blood may find its way within this tube—which is to be avoided. The bore of the stopper at *G* is about 2 cm., that of the tube *D* about 1 cm. The innermost tube is really the "van Slyke pipette." The cavity between this and the tube *D* com-

communicates with the air by another three-way tap *E*. This is for the abstraction of gas samples. If blood never gets into the tube *D* there is no chance of fouling the Haldane gas analysis apparatus. The van Slyke pipette is made of 1 mm. tubing, as also are the three-way taps. It has two bulbs *X* and *Y*, each of 1 c.c. between the graduation marks. The tube reaches, within the saturator, as nearly as possible to *M*, where it is narrowed somewhat at the tip. In the other direction after traversing the stopper, it possesses the usual tap *C*, beyond which it is produced far enough to deliver blood properly into the van Slyke apparatus.

The saturator, after evacuation, is filled in the usual way from gas reservoirs, after which it should have a positive pressure of a millimetre or two. It is then placed in water at laboratory temperature and one of the taps opened momentarily so that the gas is reduced to atmospheric pressure at a known temperature, say 15° C. Blood is injected through tap *F*—3½ c.c. should be sufficient. The dead space is cleared with mercury or nitrogen; the latter is better, as there is then no risk of mercury entering the saturator—frequently air will serve. Equilibration then takes place by rotation in the horizontal position (see description of bath later), after which the saturator, still in the equilibration tank, is placed vertically with *M* downwards. If, since the introduction of the blood, it has never been in this position before, no blood can have entered the pipette or soiled the

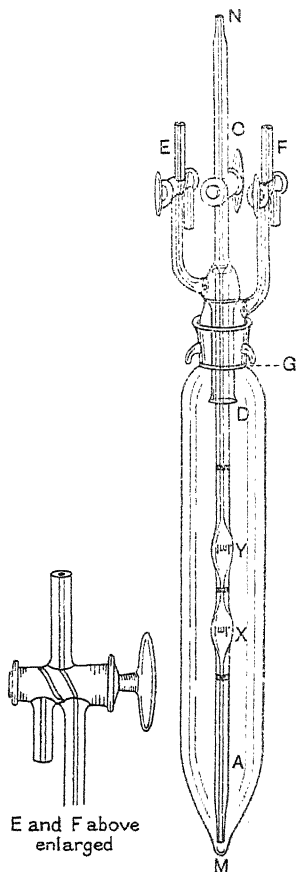


Fig. 1.

transferred to the gas analysis apparatus without opening the saturator. In practice there is no difficulty in seeing the graduations of the pipette through the walls of the saturator. The only operation which requires a word of caution is the filling of the pipette. It is necessary to open the tap very gradually and to watch the progress of the blood as it fills

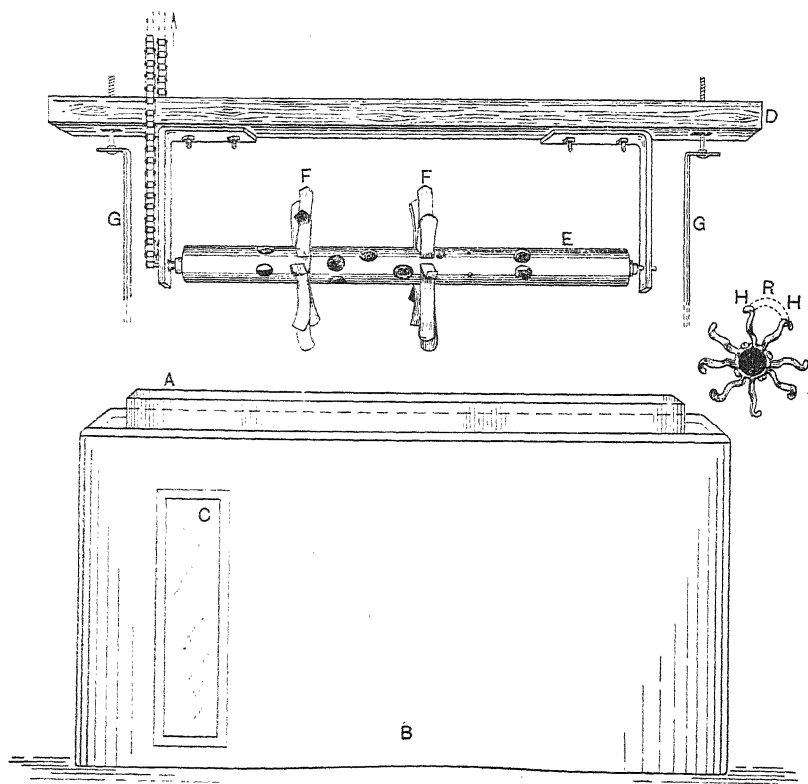


Fig. 2.

the pipette. If the blood is allowed to go in at all rapidly, especially in the smaller types of saturator to be described later, vortices of air form in the blood, and a froth appears in the pipette.

#### *Bath.*

The bath which we use is of the following design. The actual bath is a standard sized glass tank, Fig. 2, *A*, 53 × 34 × 34 cm. approximate inside measurement. This is placed in a wooden lidless box (*B*) which is

fitted with a drainage tube. The inside of the box is about one inch longer and broader than the outside of the glass tank.

Into the wood near one corner is let a window *C*, about 5 cm. broad and 25 cm. high. The saturator when in the vertical position is held in a clip attached to the glass and can be viewed through the window, so that the blood can be seen entering the pipette.

The spindle *E* on which the saturators are mounted during rotation is suspended from a stout piece of wood *D*, which rests on the top of the bath and is kept in position by a strap *G*, of sheet brass, passing round the bath. For the sake of clearness, it is shown in Fig. 2 *above* the bath, not in it.

The saturator is held in its place by spring clips, *F*, *F*. End on they are shown as *H*, *H*. The saturator is kept firmly in position by a rubber band *R*. The clips are sufficiently flexible to serve for saturators of different calibre (see below), the grip being regulated by the strength of the rubber band. String may be used.

With the bath placed inside the wooden box, the temperature can be regulated accurately enough for many purposes, by the use of an electric candle lying near the bottom of the bath fitted with a resistance outside; but of course, for accuracy over a long time a thermostat is preferable. Apart from cases in which the oxygen pressure is very low, 10 min. equilibration should suffice. In the observations necessary for ordinary oxygen dissociation curves 15 min. is long enough in all cases.

The following are five sets of duplicate analyses of the percentage saturation of hæmoglobin exposed to various oxygen pressures taken at random:

(1)	(2)	(3)	(4)	(5)
40.9	49.5	57.2	59.5	73.5
41.4	48.7	57.9	59.9	73.6

There seems to be no certain tendency for the top bulb to give a higher reading than the bottom one or *vice versa*. It had been feared that owing to the formation of dew in the bulbs the first sample of hæmoglobin solution to emerge from the pipette might be the more dilute, but this does not seem to be appreciable.

## II. SMALLER FORMS OF APPARATUS.

The saturators have been made in smaller sizes. For many purposes an apparatus of about 60 c.c. capacity has proved useful. This does not differ in principle from that described. The stopper is of the same dimensions, the taps, etc., are of rather narrower bore tubing and the bulbs of the pipette are each 0.2 c.c. capacity. The saturator from *G* to *M* is

about 11 cm. long. The pipette measures from the tip. There is no dead space. Otherwise Fig. 1 sufficiently describes it. A still smaller size, Fig. 3, has been used, of about 25–30 c.c. capacity. This demands a smaller

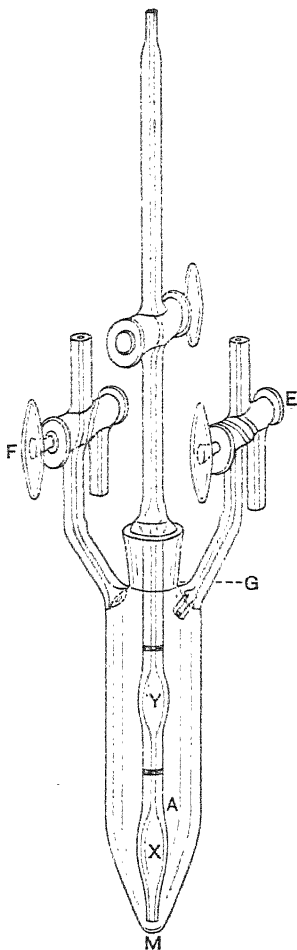


Fig. 3.

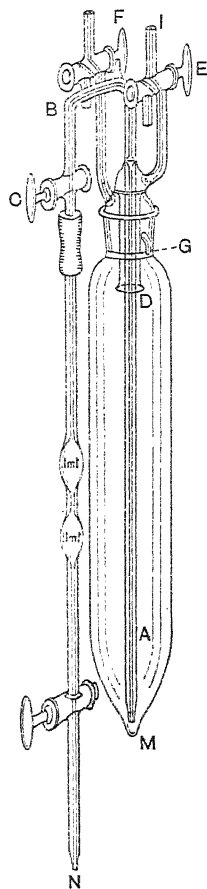


Fig. 4.

stopper and therefore a different design of apparatus, about which there is little that does not appear in Fig. 3. The bulbs are of 0.2 c.c. and the tubing from taps *E* and *F* is attached to the chamber not to the stopper, and the gas tube (connected with *E*) is continued about half a centimetre or more inside the saturator.



The following figures give the percentage saturation of five samples of blood exposed to different oxygen tensions—they are taken at random :

(1)	(2)	(3)	(4)	(5)
22.5	52.6	80.0	87.0	83.3
22.3	52.4	81.8	87.0	83.7

### III. ALTERNATIVE FORM OF SATURATOR.

Another form of saturator which gives equally accurate results is shown in Fig. 4. It was devised earlier than that shown in Fig. 1 and, though we do not now use it, there are two reasons for mentioning it. Firstly, researches were carried out with it which will be published later, and secondly, while we consider the balance of advantage to be against it, others may not take that view. The essential difference is that the measuring pipette is outside the saturator vessel to which it is attached by pressure tubing. The drawbacks are: (1) that the pipette and the attached tubing down to the orifice below *A* are filled with mercury; (2) there is the danger of a bubble of air being trapped in the rubber tubing and getting into the pipette with the blood; (3) rather more blood is necessary than in the form shown in Fig. 1. When the blood has been equilibrated and allowed to pass into the pipette, the rubber tube is cut and the pipette removed. The advantages are: (1) that the pipette is more easily read when the blood is passing into the van Slyke apparatus, and (2) that the apparatus is less easily broken.

### SUMMARY.

Forms of saturator are described, which aim at the attainment of considerable accuracy, with a less quantity of blood than is used by accurate methods already in use.

### REFERENCE.

Henderson, L. J. (1928). *Blood*. Yale University Press, New Haven.

## THE EFFECT OF DURATION OF WORK ON THE EFFICIENCY OF MUSCULAR WORK IN MAN.

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INVESTIGATIONS on the influence of the time factor on the efficiency of human muscular work have proceeded on two distinct, though by no means independent, lines, namely, the determination of the effects of rate of working and of duration of performance. Many of the early investigations showed that the rate of working materially influences the efficiency of performance, and it is therefore essential to pay due regard to this in any experimental study on the effect of duration of work on the mechanical efficiency of man which is the subject of the present investigation.

In Benedict and Cathcart's [1913] bicycle ergometer experiments with the professional cyclist subject M.A.M., the net efficiency (*i.e.* the percentage ratio of external work performed to the increased energy expenditure indicated by the oxygen used for work in excess of the resting consumption of the subject when seated on the ergometer) was found to vary with the number of pedal revolutions per minute. The maximum efficiency was attained at approximately 70 pedal revolutions per minute, but the efficiencies at slower rates were not fully investigated. It should be noted that each pedal revolution involves two complete leg movements so that at 70 revolutions per minute the contraction-relaxation time per leg movement for each leg would be 0.86 sec., although the actual duration of contraction would probably be 0.43 sec. or less, as pointed out by Garry and Wishart [1931].

Hill [1922] showed that the actual rate of change of muscle form, or speed of shortening of the muscle, is a factor influencing the energy expended in contraction, and Dickinson [1929] published an account of experiments on the efficiency of pedalling a bicycle ergometer at

varying speeds. Dickinson found that for work of short duration the excess oxygen used in work and recovery indicated a maximum net efficiency of performance when the rate was 33 pedal revolutions per minute, *i.e.* 1.82 sec. per contraction-relaxation time or approximately 0.9 sec. per contraction of the effector muscles.

Table I, compiled from Dickinson's figures for the experiments in which the load was practically constant, gives some idea of the variations in efficiency which were observed at different rates of pedalling and it may be pointed out that between 25 and 48 pedal revolutions per minute, *i.e.* when 1.2–0.63 sec. was allowed for contraction, there was relatively little variation in efficiency. Outside this range, however, the efficiency materially decreased.

TABLE I. Results of Dickinson's experiments with constant load and varying rates of pedalling bicycle ergometer.

Load (kg.)	Pedal revolutions per min.	Contraction- relaxation time (sec.)	Approximate contraction time (sec.)	Net efficiency p.c.
3.0	111	0.54	0.27	11.4
3.2	60	1.00	0.5	18.7
3.1	48	1.26	0.63	20.5
3.2	38	1.56	0.78	20.1
3.4	33	1.80	0.90	21.8
3.0	33	1.80	0.90	21.5
3.05	25	2.40	1.20	20.0
3.3	11	5.42	2.71	15.1
3.2	8	7.24	3.62	13.4

Garry and Wishart [1931] repeated some of the earlier experiments of Benedict and Cathcart in order to examine more closely the efficiencies of pedalling at the slower rates, namely below 70 pedal revolutions per minute. Their experiments differed from Dickinson's in that the excess oxygen consumption for the work was observed during the "steady state," 25 min. previous pedalling at the rate under investigation being performed before the collections of expired air were made. They found that a maximum efficiency was attained at 52 pedal revolutions per minute, namely when the contraction-relaxation time for the effector muscles was 1.15 sec., which allowed not more than 0.6 sec. for the actual duration of contraction. The optimum gross efficiencies observed were 16.3 and 18 p.c., while the net efficiencies were 2–3 p.c. higher.

Common experience teaches that the height of the saddle above the pedals must be carefully adjusted to suit the leg length of the individual if the effort of cycling is to be carried out with maximum comfort and

ease; moreover, it is not unlikely that the weight and inertia of the legs of a subject together with the linear length of the pedal stroke in relation to leg measurements and the position of the saddle in relation to the pedals may affect the optimum rate of pedalling. Again, unless the gearing and load were the same from ergometer to ergometer, the work done in starting the machine and per pedal revolution after speed had been attained would be widely different and the actual effective load per pedal revolution cause the efficiency to vary. If one could be certain that the whole of each effector muscle were in contraction for the complete duration of the down stroke in each leg movement, then perhaps more faith could be placed in comparative studies of the data obtained by different observers. However, in general terms it is quite clear that the results of investigations on human muscular efficiency all point to the conclusion that the rate of change of muscle form, or speed of contraction of the muscles, profoundly affects the efficiency of performance, and, therefore, that due regard to this should be paid in all studies of energy expenditure in muscular work when that work is executed at different rates and in varying ways.

Simonson and Hebestreit [1930] published an account of experiments on the mechanical efficiency of the human body in relation to the duration of work, and claimed to have shown that the efficiency of a man for short spells of work, *e.g.* of 1 min., is very much less than when he performs the same amount of work per minute for 3, 6 or 10 min. In some cases the efficiency was twice as great in the 10 min. spell of work as compared with the 1 min. as shown in Table II, which gives some of their results calculated in terms of percentage efficiency instead of calories per kilogram-metre.

TABLE II. Results of efficiency experiments on a horizontal pull ergometer with varying duration of work. (Calculated from the data of Simonson and Hebestreit [1930].)

Load and rate of working	Duration of work and efficiency p.c.			
	1 min.	3 min.	6 min.	10 min.
Load 10 kg. 20 pulls per min.	7.0	9.7	10.0	10.5
	6.3	11.4	12.5	13.2
	9.7	11.3	11.4	13.9
Load 10 kg. 30 pulls per min.	9.4	9.8	11.3	13.2

In their experiments, muscular work of two kinds was studied: (*a*) on an ergometer in which the subject exerted a simple horizontal pull by means of the arm muscles while standing or sitting with the spine vertical, and (*b*) stair climbing at agreed rates per minute. In both cases it would

be easy for the subject to execute the requisite number of muscular movements sharply in the minute spells and adopt a steady slower rate of muscular shortening for the long spells of work while executing the same number of pulls, or ascending the same number of stairs, per minute in each case. It is not clear from their records that the same rate of change of muscle form was ensured in the long spells of work as in the short ones.

Apart from drawing conclusions as to the significance of their results in relation to muscular work by man, Simonson and Hebestreit claimed that the changes in efficiency with prolongation of work which they observed indicated that the chemical reactions attendant on muscular contraction also changed and were therefore more complex than those put forward by Hill and others in the current theories of muscular contraction based on studies of isolated muscles. They claimed therefore that their results proved the inapplicability to man of knowledge gained by the study of isolated muscle.

Were the experiments and deductions of Simonson and Hebestreit fundamentally sound, not only would the current theories of the energetics of muscular contraction be shaken, but their results would be of considerable significance in studies of muscular work in industry. It was therefore of importance that experiments should be carried out with all possible safeguards in order to ascertain whether in actual fact fully loaded human muscles working at the same rate per minute and shortening in the same time per contraction did actually perform external work with less energy expenditure and increased efficiency in long spells of work (6 or 10 min.) as compared with similar efforts exerted in spells of short duration (1 min. or less). The following experiments were therefore devised and carried out in order to ascertain whether Simonson and Hebestreit's results could be obtained under such conditions.

#### EXPERIMENTAL PROCEDURE.

The experiments were designed so that the rate of working, the speed of shortening of the effector muscles, the total amount of work done and the load opposing the effector muscles per contraction should be the same whether the work was performed in short spells (intermittent work) or in long spells (continuous work). Moreover, the load was such that it would involve a maximal effort on the part of the effector muscles of the subject. The type of work selected was pedalling a Martin's bicycle ergometer, as this permitted accurate control of the various factors mentioned above. The attachment of a revolution counter with a loud

click to the back wheel of the ergometer enabled the subject easily to adjust and maintain his rate of pedalling so that the ticks of the metronome synchronized with the clicks of the counter.

A young man, H. L., who was a skilled cyclist and accustomed to metabolism experiments, was selected as subject. His data and the measurements of the bicycle ergometer when adjusted to his stature were as follows:

Subject H. L. Age 20.

Height 165 cm.

Weight 57.2 kg.

Total leg-length 75 cm.

Length knee to ground 43 cm.

Bicycle ergometer: Mid-saddle to pedal maximum 82 cm.

Mid-saddle to pedal minimum 47 cm.

Circumference of back wheel 144 cm.

Gear ratio 3 : 1.

As a routine the subject started resting, sitting 2 hours after a light breakfast and after 30 min. took up the work position on the ergometer. After a further period of rest in the work position a 10 min. collection of expired air was taken. The work spells, three of which were carried out each experimental session, were arranged to alternate as follows: intermittent work, continuous work, intermittent work; or continuous work, intermittent work, continuous work. Throughout each session the subject remained seated on the ergometer, and following the recovery collection, after each working effort, a resting collection of expired air was made.

The intermittent work consisted of ten short spells of 25 sec. pedalling effort, each spell being followed by rest up to 3 min. so that 250 sec. actual working time was evenly distributed over a period of 30 min. A further 15 min. was allowed for final recovery, the total expired air for 45 min. being collected in a 1000 litre Douglas bag.

The continuous work spell consisted of 250 sec. pedalling at the same rate per minute and speed of contraction as during the short spells followed by recovery up to 45 min., the whole of the expired air in work and recovery being collected. At the end of each session a final 10 min. resting collection of expired air was made while the subject was still in the work position on the ergometer.

It may be pointed out that the total oxygen cost of the effort during work and recovery must be ascertained in a study such as this, and as

the attainment of the "steady state" is impossible the whole of the expired air during work and recovery has to be collected in each case.

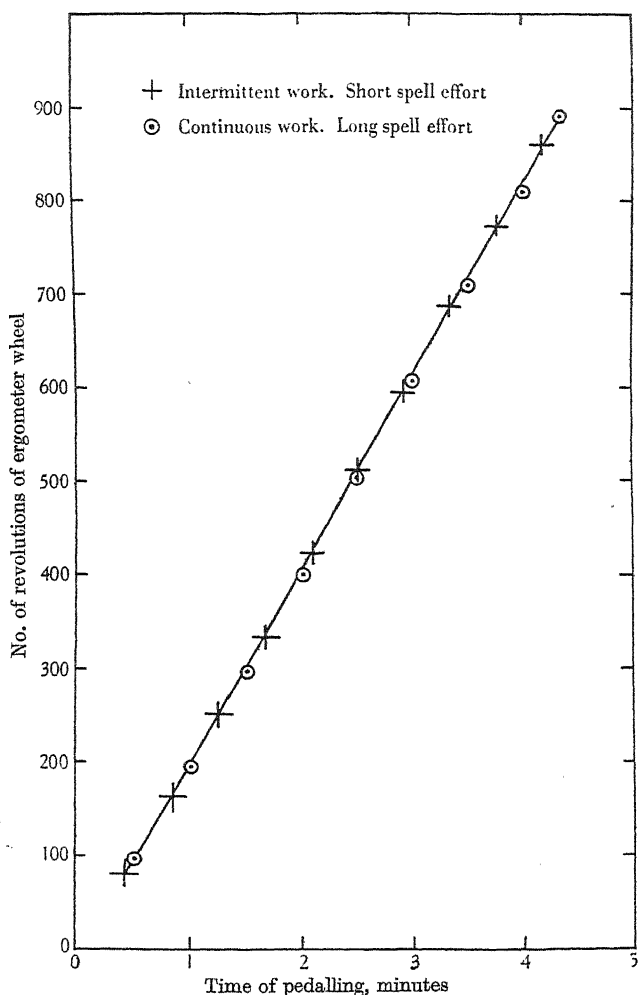


Fig. 1. Chart showing number of revolutions of bicycle ergometer wheel in ten bouts of 25 sec. effort (short-spell intermittent work), and number of revolutions observed at intervals of 30 sec. during the long-spell continuous effort of 250 sec. duration. 14. v. 31.

After every 25 sec. pedalling in the intermittent work and also during the continuous effort the number of revolutions on the counter were noted. At frequent intervals the minor variations in the spring balance attached

to the ergometer were noted, these being averaged for each experiment in order more accurately to evaluate the effective friction load due to the suspended weight. Fig. 1 shows how closely the rate of execution of the muscular movements coincided in the intermittent and continuous work spells.

In the early experiments the intermittent work involved ten starts and ten stops, while the continuous effort was executed with one start and one stop; and as it was thought that possibly starting and the negative braking effort in stopping might affect the relative efficiencies of total performance, the later continuous work experiments were carried out with one stop and immediate start after every 25 sec. of pedalling, thus making the work as nearly as possible identical in the intermittent and continuous efforts except for the actual duration of effort in relation to periods of rest.

The data for the experiments are given in Table III, which shows that in each experimental session the rate of pedalling, the total number of leg movements performed, the total amount of external work done and the work per leg movement, were as nearly as attainable the same whether the work was executed in bouts of 25 sec. effort (intermittent work), followed by rest up to 3 min., or in one continuous spell of approximately 4 min. duration.

TABLE III.

Experiment date and order	Character of work	No. of starts and stops	Total work done kg.m.	Total leg movements	Work per leg movement kg.m.	Total O <sub>2</sub> cost of work litres	Net efficiency p.c.
11. v. 31 i	Intermittent	10	5481.8	556	9.86	12.735	20.19
ii	Continuous	1	5426.2	562	9.65	14.985	16.99
iii	Intermittent	10	5339.4	558	9.57	14.467	17.32
14. v. 31 i	Continuous	1	5342.8	582	9.18	13.680	18.33
ii	Intermittent	10	5368.5	574	9.35	12.195	20.50
iii	Continuous	1	5529.9	594	9.31	13.860	18.73
27. v. 31 i	Continuous	10	5760.0	576	10.00	15.525	17.42
ii	Intermittent	10	5814.3	574	10.13	14.850	18.72
iii	Continuous	10	5822.5	586	9.94	14.670	18.62
28. v. 31 i	Continuous	7	4166.9	382	10.91	11.430	17.11
ii	Intermittent	7	4332.9	392	11.05	11.205	18.15
iii	Continuous	7	4057.8	372	10.91	9.630	19.77
29. v. 31 i	Intermittent	10	5355.7	570	9.39	11.520	21.82
ii	Continuous	10	5335.4	562	9.49	12.780	19.59
iii	Intermittent	10	5474.3	576	9.50	11.950	21.50

The rate of pedalling was 72 revs./min. throughout.

Table IV gives the net efficiencies calculated from the data in Table III and set out in order of performance of the effort whether intermittent or continuous. It is clear that whether the work done was



executed in short spells or in long spells there was little difference in the efficiency of performance.

TABLE IV. Net efficiencies in short- and long-spell work.

Short-spell inter- mittent work	Long-spell con- tinuous work	Short-spell inter- mittent work	Long-spell con- tinuous work
20-19	16-99	17-32	
	18-33	20-50	18-73
	17-42	18-72	18-62
	17-11	18-15	19-77
21-82	19-59	21-50	

The mean efficiency for the intermittent short-spell work was 19.74 p.c. as compared with a mean efficiency of 18.32 p.c. for the same amount of work when executed at the same rate of muscular movement in one continuous effort. Although the difference between the mean efficiencies cannot be regarded as significant, yet it is worthy of note that when the subject executed the work in spells of short duration his net efficiency was on the whole slightly higher than when the same total amount of work was performed in one long spell. These experimental results are therefore entirely at variance with the general claim made by Simonson and Hebestreit that their experiments proved that the efficiency of performance was approximately twice as great in long-spell effort lasting 6-10 min. as compared with that attained in work of 1 min. or less duration.

As shown in Table I above, Dickinson obtained efficiencies varying from 11.4 p.c. at a maximum rate of pedalling to 21.8 p.c. with an optimum rate, namely an order of difference approximating to the results of Simonson and Hebestreit. Further experiments were therefore carried out in order to ascertain whether such a variation in efficiency could be demonstrated when the subject performed the same total amount of work in short-spell bouts at a maximum rate and in long-spell work at an optimum rate.

In these experiments the subject did 6 min. work at a rate of 0.9 sec. per leg movement—this being the long spell; the short-spell effort was for 6 min., each minute being made up of 20 sec. pedalling at a maximum rate of 0.29 sec. per leg movement followed by rest up to 1 min.

As shown in Table V the efficiency of performance during the short-spell effort was from 5 to 7 p.c. less than that attained during the long spell.

It is apparent from the data given in Table V, and from the well-known influence of variations in the speed of muscular movement on

TABLE V.

	Long-spell continuous work at optimum rate: 0.9 sec. per leg movement	Short-spell intermittent work at maximum rate: 0.29 sec. per leg movement	Long-spell continuous work at optimum rate: 0.9 sec. per leg movement
No. of starts and stops	6	6	6
Rate of pedalling: revs./min.	33	101	33
Total work done: kg.m.	3571.5	3823.5	3758.5
Total leg movements	382	406	404
Work per leg movement: kg.m.	9.35	9.41	9.30
Total O <sub>2</sub> cost of work: litres	7.530	11.580	8.370
Net efficiency p.c.	22.25	15.51	21.07

efficiency, that the results of Simonson and Hebestreit may be, in part, accounted for by more rapid change in muscle form in work of short duration than in long-spell work.

Hill [1932] drew attention to this possible explanation of their results in 1931, and since that date Simonson [1933] has re-examined his data and carried out further experiments in which he claims to have substantiated his former results and to have proved that variations in speed of movement could not account for the changes in efficiency which he has observed. In the later experiments Simonson and Sirkina [1933] employed a different type of effort, namely lifting dumb-bells varying in weight from 2 to 8 kg. In these experiments the subject first stood with arms by the side and then lifted the dumb-bell with extended arm until level with the shoulder. The lifting and lowering efforts were carried out at the same speed. Rates of 10 or 20 lifts per minute were studied and the duration of effort varied from  $\frac{1}{2}$  to 7 min. With this type of effort and with the lightest loads he found wide variations in efficiency in relation to the duration of the work. In some cases the efficiency attained in the 7 min. spell was nearly 6 times as great as that for the  $\frac{1}{2}$  min. spell of similar work. Simonson gives his results in terms of small calories energy expended per kilogram-metre of work done, but this method of presentation obscures the fact that the type of work he selected was performed by his subjects at remarkably low efficiencies as shown in Table VI which gives some of his data and the actual efficiencies as calculated from them.

It is well established that purely dynamic muscular effort in man can be performed with an efficiency of the order of 25 p.c., but if the character of the work involves postural strain or static effort, then the expenditure of energy per unit of external work as measured is proportionately increased and the efficiency of man as a whole is lessened. Such low efficiencies as 0.83, 1.98, or 4.93 p.c. are probably due to a relatively large

TABLE VI. Efficiencies for dumb-bell lifting. (Calculated from Simonson's data [1933].)

Load kg.	Lifts per min.	$\frac{1}{2}$ min. spell		7 min. spell		Efficiency ratio $\frac{1}{2}$ min./7 min.
		cal./kg.m.	Efficiency p.c.	cal./kg.m.	Efficiency p.c.	
2	10	280	0.83	47.5	4.93	5.9
4	10	202	1.16	38.6	6.07	5.24
6	10	118	1.98	38.3	6.12	3.08

expenditure of energy by static effort of some kind, and it is of interest to note that when Simonson employed heavier dumb-bells, or increased the number of lifts per minute, he did not get such remarkable changes in efficiency in relation to the duration of the effort, and that the efficiency in the initial short-spell effort was higher.

Simonson's figures show that load and speed of movement in work of this character affect the efficiency profoundly. For example, with a 6 kg. dumb-bell lifted in 0.75 sec. and lowered in 0.75 sec., for a period of  $\frac{1}{2}$  min. (10 movements), the efficiency was 4.17 p.c., but with a 2 kg. dumb-bell lifted in 3 sec. and lowered in 3 sec. for a period of  $\frac{1}{2}$  min. (five movements) the efficiency was only 0.83 p.c., *i.e.* one-fifth of the former efficiency although the duration of work was the same in both cases.

It is evident that Simonson's data, on the basis of which he claims to have established a two to sixfold increase in efficiency in work of 7 min. duration as compared with the efficiency for work lasting  $\frac{1}{2}$  min., were obtained using a type of work involving postural strain, static effort, submaximal loads and the execution of the muscular movements at suboptimal rates. Moreover, the increased efficiency ratio is calculated from initial experiments in which the expenditure of energy on dynamic muscular effort was very small indeed.

In discussing the method and results of the bicycle ergometer experiments described above as briefly referred to by Hill [1932], Simonson [1933] not only raises objection to the use of this ergometer but states that the time interval of rest up to 3 min. allowed for recovery after each 25 sec. pedalling was insufficient, and that to obtain his results 30 min. should be allowed between successive bouts of short-spell effort. It is quite true that in the bicycle ergometer experiments a number of short spells of work were carried out instead of one short spell only as employed by Simonson, but this modification of experimental routine was introduced for two reasons, namely to ensure that there would be a reasonably large increase in oxygen consumption

due to the work, and to enable the same total amount of work to be executed in the short-spell bouts as a group as in the long-spell effort.

In view of Simonson's contentions further experiments on the bicycle ergometer have been carried out and every effort has been made to obtain his results by using submaximal loads, very slow rates of pedalling and a single short-spell effort of 1 min. The same subject was used as for the earlier experiments. The whole of the expired air during work and recovery for the 1 min. and 6 min. spells was collected as before, but in the case of the short-spell effort two Douglas bags were used, the expired air for the 1 min. work and 3 min. recovery being collected in one bag while the second bag was used for 26 min. further recovery.

It will be seen from the data of these experiments as shown in Table VII that Simonson's results were not confirmed, for the mean efficiency for work of 1 min. duration was 17.86 p.c. as compared with 18.19 p.c. for precisely the same type of effort when continued for 6 min.

TABLE VII. Efficiencies for short- and long-spell work on the bicycle ergometer.

	Short-spell work 1 min.	Long-spell work 6 min.	Short-spell work 1 min.
No. of starts and stops	1	1	1
Rate of pedalling: revs./min.	20	20	20
Total leg movements	40	240	40
Total work done: kg.m.	152.9	912.38	153.79
Work per leg movement: kg.m.	3.82	3.8	3.84
Total O <sub>2</sub> cost of work: c.c.	442	2165	370
Net efficiency p.c.	16.23	18.19	19.5

It should be noted that in the experiments detailed in Table VII the total work done in 1 and 6 min. respectively closely corresponded in amount to that done by Simonson's subjects in the experiments on the horizontal pull ergometer, Table II above; but whereas Simonson's 1 min. spell showed an efficiency of 6.3 p.c. an efficiency of 16.23 p.c. was attained on the bicycle ergometer. It is possible, therefore, that static and postural components in Simonson's effort may partly account for his results.

#### DISCUSSION.

In all the experiments which have been carried out on the bicycle ergometer in which uniformity has been ensured in load, rate of working and speed of muscular movement, no material difference in efficiency has been observed whether the work was performed in short spells of 1 min. duration or less, or in spells of work of 6 min. duration.

The results of this investigation do not support the conclusions of Simonson and Hebestreit who claim to have shown by experiments on a horizontal pull ergometer and dumb-bell lifting that a two to sixfold increase in efficiency occurs in man when the duration of muscular effort lasts for 6 or 10 min. as compared with similar work executed for  $\frac{1}{2}$  or 1 min. Simonson contends that the type of effort involved in pedalling a bicycle ergometer is not suitable for demonstrating his results, but it is difficult to understand why, if his phenomenon is, as he claims, a fundamental characteristic of muscular work in man, it should not be demonstrable on a machine such as the bicycle ergometer on which the additional effort occasioned by the work is mainly dynamic and expended by the effector muscles themselves with almost a minimum of postural strain. It would appear from what has already been said that the type of work selected by Simonson is not suitable for the study of the fundamentals of dynamic muscular effort in man, and therefore that the results of his experiments do not constitute sufficient justification for him to advance criticisms of existing theories of the intimate metabolic processes involved in muscular contraction, or to make generalizations in regard to the effect of duration of work on efficiency.

Although it is necessary to criticize Simonson's deductions on these grounds it is not thereby intended to throw any doubt on the accuracy of his experimentally determined data, for if his results are considered in the light of recent work on muscle it is apparent that they are precisely what would be predicted as likely to be found in experiments of the nature carried out by him.

Adrian and Bronk [1928, 1929] showed that increasing strength of contraction (lifting the heavier dumb-bells in Simonson's experiments) is the result of increased frequency of motor impulses leading to a fused ~~tetanus~~ together with the employment of a larger number of neuromuscular elements in some cases. Bronk [1930] showed by a series of experiments on the sartorius of the frog that it is possible for a muscle to exert a continued force more economically if separate muscle twitches give place to a fused tetanic contraction, and that the economy in energy expenditure increases with the degree of fusion of the twitches until complete fusion is reached. The increase in economy, or in the efficiency of production of "tension-time" amounted to three or fivefold with increasing fusion of twitches due to increased frequency of stimulation, and it is of interest to consider this result in connection with Simonson's experiments, quoted above, in which in the case of a light load (2 kg. dumb-bell lifted slowly) the efficiency was 0.83 p.c., while for

a 6 kg. dumb-bell lifted four times as rapidly but for the same duration of work,  $\frac{1}{2}$  min., the efficiency was 4.17 p.c., *i.e.* five times as great.

However, the main point at issue in the present discussion is the variation in efficiency in relation to duration of effort, and here again a clue to the explanation of Simonson's two to sixfold increase in efficiency is to be found in the recent studies on the heat production and economy of energy expenditure in maintaining muscle contraction. Bronk [1930] recalled the fact that the earlier work of Hartree and Hill [1921] indicated that the heat production at the commencement of a contraction is relatively greater than at later periods during its maintenance. From the point of view of the economy of production of tension time in relation to duration of stimulation Bronk investigated the well-known characteristic of muscle that prolonged stimulation at a uniform frequency initially below that required to produce summation leads finally to a complete tetanus. Working with the sartorius of the frog he found that with the increasing fusion of twitches as the stimulus was prolonged the economy in maintaining muscular tension also increased until at the end of a 2 min. period of stimulation at a frequency of 4 per second more than a fivefold increase in economy was recorded.

Bozler [1930] working with the retractor muscle of the pharynx of the edible snail obtained results similar to those of Bronk and showed that the economy of maintaining tension in long contractions increases six to ten or even fifteenfold following a few minutes' moderate exercise. More recently Bronk [1932] carried out experiments on crustacean muscle, and with the adductor muscle of the crab proved that the increase in economy of maintaining contractions is due to the slowing of the muscle as a result of previous contractions. This slowing of the muscle and prolongation of relaxation time permits summation and fusion of twitches in spite of a very low frequency of motor impulses, and with increasing fusion of twitches the economy of maintaining the contraction increases four or fivefold.

The results of these investigations have an important bearing on problems concerning the maintenance of postural tone and the prolonged contractions involved in static effort. On the other hand, in rapid dynamic effort, involving little postural strain or static effort, as in the case of rapid pedalling on the bicycle ergometer, the changes produced by prolonged activity in the time relations of contraction would have little influence, except on the optimum speed. Inasmuch as the type of work studied by Simonson involved a large—indeed a predominant—static component, ample opportunity was afforded for the slowing of muscle

contractions resulting from previous activity to increase the efficiency. It seems reasonable therefore to ascribe the increase in efficiency with prolongation of effort which he observed to the presence of relatively large static and postural components in the work. If this explanation of Simonson's results be true, then his experiments serve to confirm in man the teachings of studies of prolonged contraction in isolated muscles, but it would necessarily follow that any generalization from them as to the increased efficiency of muscular work in man with prolongation of effort must be limited to types of muscular work in which static and postural components predominate.

#### SUMMARY.

Experiments have been made in order to ascertain whether the duration of effort affects the efficiency of muscular work in man when the load, rate of working and speed of shortening of the effector muscles are carefully controlled.

The type of work selected was pedalling a Martin's bicycle ergometer in which the muscular effort is primarily dynamic.

The total excess oxygen used for work and recovery (*a*) in short-spell efforts of 25 sec. to 1 min. duration, and (*b*) for 4 or 6 min. similar work, has been determined and the net efficiencies of performance calculated.

No material difference in efficiency was found whether the duration of work was 1 min. or less or 6 min., and it is concluded that provided the effort is primarily dynamic and the speed of muscular movement, load and rate of working are the same, the actual duration of work has little if any effect on the net efficiency of performance.

The results of this investigation are in accordance with predictions based on previous work, but they are in complete disagreement with the contentions of Simonson and Hebestreit who claim to have shown that a two to sixfold increase in efficiency occurs in man in muscular work of 6 to 10 min. duration as compared with similar effort exerted for 1 min. or less.

It is suggested that the predominant static and postural components in Simonson and Hebestreit's experiments accounted, at least in part, for their results, particularly in view of the known fact that the economy of maintaining a prolonged contraction is increased by previous activity.

I am greatly indebted to Prof. A. V. Hill for his helpful criticism and interest.

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## ACTION OF ACETYLCHOLINE ON THE BRAIN AND ITS OCCURRENCE THEREIN.

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THE fact that the parasympathetic and the sympathetic nerves produce their effect by a specific chemical substance has been demonstrated by several workers. Dixon [1906], Howell [1906] and Sherrington [1925] suggested this possibility, while the work of Loewi [1921] on the amphibian heart definitely showed that parasympathetic stimulation liberated a substance resembling acetylcholine, and sympathetic stimulation liberated a substance resembling adrenaline. The work of Dale and Gasser [1926] on the denervated mammalian muscle and the subsequent demonstration of acetylcholine in the normal tissues by Dale and Dudley [1929] considerably helped in establishing the theory of humoral transmission. Literature concerning the theory of humoral transmission has been discussed by Dale in his Croonian lecture [1929], and the account given there shows that most of the work done has been concerned with the peripheral terminations of the nerves. Evidence is presented in this paper which suggests that humoral transmission may occur in the central nervous system.

Cushing [1931] found that injections of pituitrin and of pilocarpine into the brain ventricles produced similar effects, although these two drugs produced totally different effects when given intravenously. He suggested that the drugs when given intraventricularly stimulated centres situated in the wall of the ventricles. This work suggested the possibility of vago-mimetic drugs stimulating the vagal centres when injected intraventricularly, and the writer made experiments to compare the effects produced by central stimulation of the vagus and intraventricular injection of acetylcholine.

Vagal control of respiration has been a subject of considerable experimentation, and there is a certain degree of unanimity about the

effects produced by stimulation of the central end of the vagus on respiration. Rosenthal [1865] first showed that stimulation of the central end of the vagus caused inhibition of respiration, and many other workers have confirmed this observation since then. The effects produced depend on the strength of the stimulus; weak stimuli cause an inhibition and sometimes an acceleration of respiration, but a sufficiently strong current always produces inhibition.

#### METHODS.

Cats were used in all experiments. The animals were anaesthetized with paraldehyde (1 c.c. per kg.) and ether or urethane (1.8 g. per kg.). Respirations were recorded by a tracheal cannula connected to a tambour and the blood-pressure by a cannula in the carotid artery. The vagi on both sides were dissected and cut in the middle of the neck. It was found that electrical stimulation of the central end of the cut vagus always caused a complete cessation of respiration if a sufficiently strong stimulus was used. Acetylcholine was injected into the ventricles through a small hole trephined at the junction of the coronal and sagittal sutures. Actions produced by the mechanical effects of the injection were controlled by injecting an equal volume of saline before and after the acetylcholine injections.

#### EFFECTS OF INTRAVENTRICULAR INJECTIONS OF ACETYLCHOLINE.

Fig. 1 shows the effect of an intraventricular injection of 0.5 $\gamma$  of acetylcholine. This produced a complete inhibition of respiration for about 30 sec. The drug given by this route produced no certain effect on the blood-pressure, for the slight but long continued fall in blood-pressure observed was probably due to the mechanical effects of cessation of respiration. The effects produced by intraventricular injections of acetylcholine in experiments on thirty cats are summarized in Table I. Table I shows that there is an extensive individual variation in the response to acetylcholine as regards the amount needed to produce an effect. The effects produced by acetylcholine were completely reversible, and several injections could be given to a single animal. In only two cases out of thirty-two did the animals fail to respond to a dose of 2 $\gamma$  of acetylcholine.

In a few experiments injections of less than 0.5 $\gamma$  acetylcholine produced acceleration of respiration, and in such cases it was found that stimulation of the central end of the vagus also produced augmentation of respiration.

A few experiments were made in which any secondary effects due to interference with ventilation were eliminated. Movements of the diaphragm of cats were recorded by a modification of the technique described by Thomas and Frank [1928].

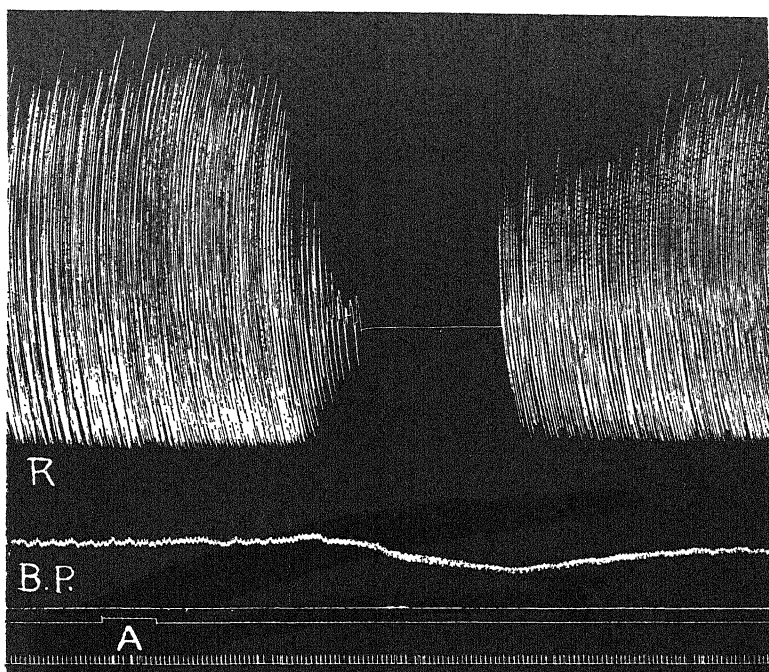


Fig. 1. Cat's respiration (R) and blood-pressure (B.P.). At A 0.5 $\gamma$  of acetylcholine injected in the right lateral ventricle. Time, 2 sec.

TABLE I. Effects produced by intraventricular injections of acetylcholine.

Dose of acetylcholine in $\gamma$	Incidence of effects		
	Cessation of respiration for 10 sec. or more	Depression and slowing of respiration	No effect on respiration
0.05-0.5	2	17	4
0.5-1.0	4	1	2

Under artificial respiration a strip of diaphragm about 1 cm. wide and 4 cm. long was isolated in such a way that the phrenic nerve ran along the centre of the strip. The vessels accompanying the nerve were isolated and cut, but the nerve was left intact. The lower end of the strip was fastened to a fixed rod and the upper end connected to a writing lever. The strip,

if moistened with Locke's solution, contracted regularly for a considerable time.

Fig. 2 shows a typical record of an experiment. This figure shows that

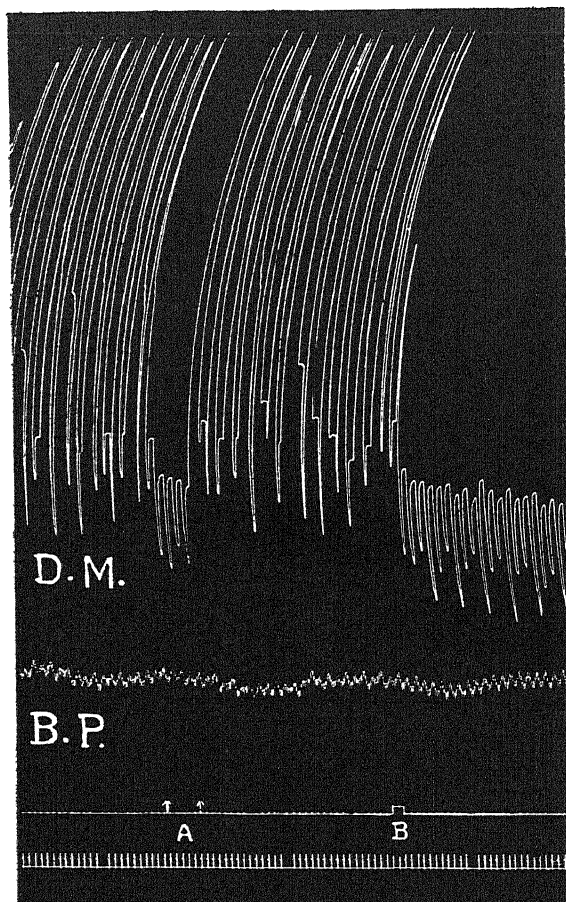


Fig. 2. Cat's diaphragm strip movements (D.M.) and blood-pressure (B.P.). At *A*, Stimulation of the central end of the left vagus. *B*, Injection of 0.1  $\gamma$  of acetylcholine in the third ventricle. Time, 2 sec.

the action of intraventricular acetylcholine on respiratory movements occurs when artificial respiration is maintained and when the muscle is isolated from circulation. Therefore the drug must act on the central nervous system.

Small doses of atropine (0.5 mg. per kg.) suffice to abolish the effects produced by intravenous injections of acetylcholine and the latter are augmented by small doses of physostigmine (0.1 mg. per kg.). The inhibition of respiration produced by intraventricular injections of acetylcholine is not affected by atropine when the latter drug is given either intravenously or intraventricularly. Furthermore, atropine does not affect the inhibition of respiration produced by stimulation of the central end of the vagus.

Atropine was given in doses of 0.1–1 mg. intravenously (seven experiments) and 0.02–0.1 mg. intraventricularly (three experiments). In no case was any change observed in the effects produced by intraventricular injections of acetylcholine (0.1–0.5  $\gamma$ ), given before and after the atropine.

The effects of stimulation of the central end of the vagus were observed in thirty experiments before and after similar doses of atropine, and in no case did this drug cause any change in the effects produced.

Engelhart and Loewi [1930] showed that physostigmine inhibited the hydrolysis of acetylcholine by an esterase commonly present in the blood. If therefore the inhibiting action on respiration seen after stimulation of the central end of the vagus is due to liberation of acetylcholine, it might be expected to be enhanced by intravenous and intraventricular injections of physostigmine. It was however found that neither intravenous nor intraventricular injections of physostigmine materially altered the effects of vagus stimulation or produced any other detectable change in the effects of intraventricular acetylcholine.

Physostigmine was given in doses of 0.1–2 mg. intravenously to ten animals, and intraventricularly to fourteen animals. In no case was any change observed in the effects produced before and after these injections by either central stimulation of the vagus or by intraventricular injections of acetylcholine.

These experiments show that the respiratory inhibition produced by intraventricular administration of acetylcholine is neither paralysed by atropine nor augmented by physostigmine, and that the same is true of the effects produced by stimulation of the central end of the vagus.

There are, however, various peripheral actions of the vagus and of acetylcholine which are not antagonized by atropine. For example, atropine does not abolish the augmentor effect of the vagus on the intestinal movements. Similarly the vaso-dilator effect produced by antidromic stimulation of the sensory nerves is not affected by atropine, although this abolishes the vaso-dilator action of acetylcholine. The anomalies observed in regard to the antagonism of acetylcholine by

atropine have been discussed by Dale [1929]. Dale and Gaddum [1930] observed that the action of acetylcholine on denervated mammalian muscle is not abolished by atropine. Dale [1929] in his general discussion pointed out that, in various other cases, effects produced by nerve stimulation which mimicked acetylcholine action showed a similar difference as regards the effect of atropine. He concluded that the failure of atropine to paralyse the vaso-dilator effect of antidromic stimulation of sensory nerves did not disprove the hypothesis that this effect was due to liberation of acetylcholine.

The experiments described above show that intraventricular acetylcholine and central stimulation of the vagus produce very similar inhibition of respiration. The simplest explanation is to assume that central stimulation of the vagus liberates acetylcholine, and this theory is not disproved by the failure of atropine and physostigmine to influence the response, because these drugs do not influence the response of intraventricular administration of acetylcholine.

Effects of stimulation of the central end of the vagus depend upon the strength of the stimulus. With a weak current it is not unusual to find that, during the period of stimulation, there is actually an increase in the rate as well as depth of respiration and the action of inhibition comes on after cessation of the stimulus. In animals with normal respirations the apnoea might conceivably be a physiological one following hyperpnoea. If, however, this effect is studied in animals under artificial respiration whose respiratory movements are studied by the diaphragm strip method, it becomes obvious that the apnoea is a true effect of vagal stimulation. It is easier to explain these delayed effects by a secretory theory than by any other, for it can be argued that during the period of stimulation a chemical substance is liberated which persists after cessation of the stimulus. The increase in the rate and depth of respiration may be ascribed to the immediate effect of stimulation of an afferent nerve, while the delay in the cessation of respiration which occurs after cessation of the stimulus is an effect that suggests the liberation of an active chemical substance.

Beattie, Brow and Long [1930] observed that stimulation of the central end of the vagus in cats under light chloroform anaesthesia precipitated irregularities in hearts which were beating regularly before the nerve stimulation. In a certain proportion of cases I have observed irregularities of the heart precipitated after stimulation of the central end of the vagus. Injections of small doses of acetylcholine (0.1-0.5 $\gamma$ ) in the lateral ventricle of such animals also produced irregularities of the heart.

It would appear from the experimental data given above that there is some reason to believe that an acetylcholine-like substance might be liberated in the brain during stimulation of the central end of the vagus. If it is liberated in the brain it should eventually appear in the cerebro-spinal fluid. Attempts were therefore made to see if any such substance could be detected in the cerebro-spinal fluid after stimulation of the central end of the vagus.

#### OCCURRENCE OF ACETYLCHOLINE IN THE CEREBRO-SPINAL FLUID AND THE BRAIN.

Cats were used for these experiments, artificial respiration was employed and the central end of the vagus was stimulated with a current sufficiently strong to produce a cessation of respiration. Immediately after or during the nerve stimulation, the cisterna magna was tapped by a medium-sized needle and about 1 c.c. of clear cerebro-spinal fluid withdrawn. Another sample was collected about 20 min. later, and the two samples were tested on the blood-pressure of cats and on isolated ventricular strips of the frog's heart, to determine whether any acetylcholine-like substance was present. The object of these experiments was to determine whether a greater amount of depressor substance was present in the cerebro-spinal fluid after stimulation of the central end of the vagus than was present in the control specimen. The simple comparative nature of this estimation makes it unnecessary to discuss the nature of the effects produced by the injections.

Injections of normal cerebro-spinal fluid intravenously produce very varying results on the blood-pressure of cats, and the exact significance of the results obtained from such experiments is therefore obscure. In these experiments only the comparative action was taken into consideration to see if any depressor substance was liberated in the cerebro-spinal fluid after central vagal stimulation when no such substance could be detected in the normal cerebro-spinal fluid, or if any depressor substance was present in the normal whether its quantity was enhanced after vagal stimulation.

Thirty experiments were made, and in twenty-seven no certain difference was observed as regards the vaso-depressor actions produced by the intravenous injections of the two samples of cerebro-spinal fluid. In two cases the sample collected after vagal stimulation produced the greater action, but in one case the control sample showed the greater action.

The cerebro-spinal fluid also was tested for acetylcholine upon the frog's heart. The fluid was diluted with half its volume of water and was applied to isolated ventricular strips according to the method used by Clark [1926]. Twenty-one experiments were made and the results were negative in nineteen cases, but in two cases the cerebro-spinal fluid obtained after vagal stimulation produced a depressor action, which was abolished by atropine, although the control sample produced no effects.

The tests on cats' blood-pressure and on the frog's heart should detect the presence of about 0.01% of acetylcholine in 1 c.c. (1 in 100,000,000). The general result of the tests is to show that in the great majority of cases the acetylcholine content of the cerebro-spinal fluid, both before and after vagal stimulation, is less than 1 in 100,000,000, but that occasionally vagal stimulation causes the appearance in the cerebro-spinal fluid of an acetylcholine-like substance with an action equivalent to a concentration of acetylcholine of about 1 in 100,000,000.

Attempts also were made to demonstrate the presence of acetylcholine in the brain.

Since the observation of Schäfer and Moore [1896] that intravenous injections of brain extracts produce a fall in blood-pressure, there has always been a controversy about the nature of the substance in those extracts. Mott and Halliburton [1899] thought it to be choline, while Vincent and Sheen [1903] did not think it to be so. The controversy narrowed to a smaller issue after some time, the point of discussion being whether the "important" substance present was choline or not. Apart from pharmacological tests acetylcholine was demonstrated in the brain extracts by chemical methods also [Gulewitsch, 1899]. Later researches [Miller and Miller, 1911; Major and Weber, 1929, 1930; Major, Nanninga and Weber, 1932; Euler and Gaddum, 1931] have shown that there are several substances present in the brain which have a depressant action on the blood-pressure. Chang and Gaddum [1933] estimated the acetylcholine equivalent of dog's brain as 0.4% per g. of tissue. This value is lower than those found for many other tissues of the dog such as the intestines and the auricle.

The author [1933] estimated the acetylcholine content of the brain by the following method.

Fresh brains of cats and rabbits were used for this purpose. The extracts were prepared by macerating different parts of the brain in an acetone-water-acid mixture containing 75 p.c. acetone, 15 p.c. distilled water and 10 p.c. *N*/100 acetic acid. The emulsion was allowed to stand for 24 hours at 5° C., filtered and evaporated to dryness *in vacuo* at room temperature.



The residue was dissolved in distilled water and used for injections as crude extract.

The pharmacological properties of this extract were found similar to those of acetylcholine. It lowered the blood-pressure after an intravenous injection, and the nature of the fall so produced was indistinguishable from that produced by acetylcholine. It produced contraction of the isolated gut of the rabbit, depressed the perfused frog's heart and inhibited the respiration of the cat after an intraventricular injection, in the same manner as acetylcholine.

The acetylcholine equivalent of the extracts was estimated by measuring the fall in cat's blood-pressure produced by intravenous injections. Results obtained are summarized in Table II. The value obtained in the case of the cat agrees with that obtained by Chang and Gaddum [1933] in the dog.

TABLE II.

Animal	No. of expts.	Acetylcholine equivalent of extracts of the basal ganglia in $\gamma$ per g. fresh tissue	
		Range of figures	Mean
Cat	10	0.2 -1.3	0.47
Rabbit	4	0.04-1.0	0.15

Extracts were also made of the cerebral cortex and cerebellum. The cortical extracts were only slightly weaker than the basal ganglia extracts, but the cerebellar extracts showed only half this activity.

The nature of the depressor substances present in the brain has been discussed by Euler and Gaddum [1931] and the general principles regarding biological demonstration of acetylcholine by Chang and Gaddum [1933]. These workers have shown the multiplicity of biologically active substances present in tissue extracts. Their work shows that the following tests for acetylcholine deserve special consideration: (1) production of a vaso-depressor action which is abolished by atropine, and (2) production of a contraction of the isolated rectus abdominis of the frog which is augmented by physostigmine.

Dr Stedman kindly provided me with a purified acetylcholine esterase isolated from horse blood [1932]. This esterase breaks down acetylcholine, its action is rapid for it completely destroys acetylcholine in high dilutions in less than 5 min., and as far as is known its action is specific for choline esters. This substance therefore provides a simple method for testing the presence of acetylcholine in extracts. By itself it has no detectable pharmacological action on blood-pressure or respira-

tion, and produces no alteration in the records of these so as to interfere with quantitative estimations. Another advantage of this method is that injections of extracts and acetylcholine can be repeated in the same animal and results obtained thus properly controlled. I have used the following technique to demonstrate acetylcholine in the brain extracts.

The extract to be tested is divided in three equal portions. The first is injected intravenously and the effects on the blood-pressure noted. The esterase is added to the second and, after about 5 min., this is injected intravenously as before. The third is used as a control to see if the response is the same as the first one. Usually the first and third injections show the same degree of vaso-depression while the second shows either no fall of blood-pressure at all or a comparatively smaller fall.

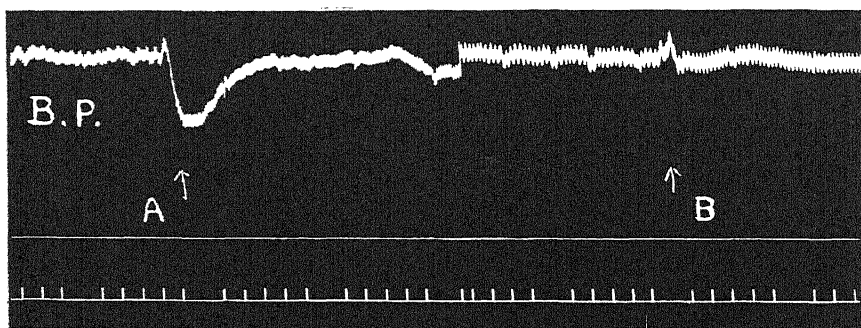


Fig. 3. Cat's blood-pressure (B.P.). A, 0.5 c.c. of basal ganglia extract given intravenously B, 0.5 c.c. of the same extract treated with esterase before administration. Time, 10 sec.

Brain extracts tested in this way show that the relative quantities of acetylcholine and the other depressor substances present in the brain vary a good deal. It is not unusual to find an extract whose depressor activity is completely abolished by treating it with the esterase. Fig. 3 shows the results obtained in one such experiment. The first injection produced a fall of blood-pressure which was completely abolished by the action of the esterase.

I have obtained about the same results with atropine. Atropine abolishes the depressor activity of some extracts only partially and of others completely. Fig. 4 shows the complete antagonistic action of atropine on the depressor effect of the basal ganglia extract.

The extracts were also tested on the isolated rectus abdominis of frogs (*R. temporaria* and *esculenta*). Application of extracts to these produced

a contraction which was markedly enhanced by soaking the muscle in a 1:100,000 solution of physostigmine. This effect is a specific test for acetylcholine, and it can be concluded from all these experiments that one of the depressor substances present in the brain extracts is acetylcholine.

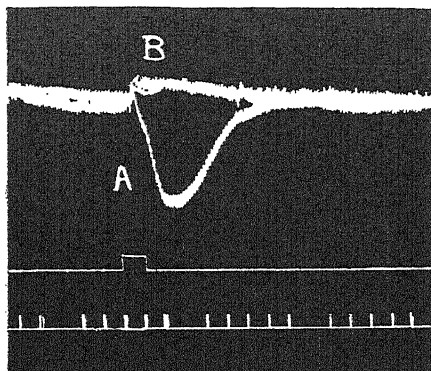


Fig. 4. Cat's blood-pressure. Drum turned back to get superimposed tracings. 0.5 c.c. of basal ganglia extract injected twice at the signal. *A*, Tracing before intravenous atropine. *B*, Tracing after atropine. Time, 10 sec.

#### DISCUSSION.

The presence of an acetylcholine-like substance in the brain, its higher concentration in the basal ganglia which is supposed to be the seat of a number of visceral activities and its occasional occurrence in the c.s.f. after vagal stimulation appear to be of some significance when viewed along with the fact that stimulation of the central end of the vagus, and injections of small quantities of acetylcholine in the ventricles of the brain have a similar action on respiration. The delayed effects of vagus stimulation and the occasional production of cardiac irregularities after vagal stimulation and intraventricular acetylcholine injections also support the view of humoral transmission in the central nervous system. The evidence so far collected in support of the theory of humoral transmission is chiefly indirect, the only direct evidence being the occasional occurrence of a vaso-depressor substance in the c.s.f. after vagal stimulation when no such substance could be detected in the normal c.s.f. It has however been possible to present certain data which suggest that such a transmission is possible.

## SUMMARY.

1. Injection of acetylcholine (0.1-1.0 $\gamma$ ) into the cerebral ventricle of cats causes effects similar to those produced by stimulation of the central end of the vagus, namely arrest of respiration and, occasionally, cardiac irregularity.

2. Intraventricular injections of acetylcholine (0.1-1.0 $\gamma$ ) do not produce a fall of blood-pressure.

3. The effects produced by intraventricular injections of acetylcholine are not affected by administration of large doses of atropine or of physostigmine.

4. Extracts of the basal ganglia contain a depressor substance which shows the properties of acetylcholine in a variety of biological tests. The acetylcholine equivalent of this substance is 0.4 $\gamma$  per g. of basal ganglia in cats.

5. The cerebellum and cortex contain less of this substance than the basal ganglia.

I desire to acknowledge my indebtedness to Prof. A. J. Clark for suggestions and constant help throughout the course of these experiments. The expenses of the research were defrayed by a grant from the Moray Fund of Edinburgh University.

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## ESTIMATION OF FIBRINOGEN AND THROMBIN.

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THE objective of the experiments about to be described is the estimation of fibrinogen and thrombin. The former is estimated in terms of the amount commonly present in human blood plasma to which 0.3–0.7 p.c. of trisodium citrate has been added: this amount will be shown later to be remarkably constant. The source of thrombin is the venom of *Echis carinatus*, a small Indian viper; the unit of concentration of thrombin is taken as that concentration which will completely convert into fibrin the fibrinogen contained in normal human plasma.

The methods employed for estimation are based upon the effect on fibril formation of diminution of one or other component of the coagulant mixture [Barratt, 1915]. The term "coagulation time" is used in this investigation to signify the time of onset of coagulation indicated by the first appearance, under dark-ground illumination, of fibrils of fibrin. For dilution of plasma or venom 0.85 p.c. NaCl solution was employed. The temperature of experiment was 16–18° C.

In the observations given in Tables II and III equal parts of citrated plasma and venom solution were taken, in all other observations the proportion was nine of the former to one of the latter. The actual concentration of plasma and venom present in the mixed liquid in each observation is given in the tables in the second and third columns respectively.

*Estimation of fibrinogen.*

If equal amounts of thrombin are added to a series of increasing dilutions of fibrinogen, contained in citrated human plasma, the coagulation time is found to increase with each successive dilution, as is illustrated by the experiment set forth in Table I and by the curve to the

TABLE I. The effect of dilution of fibrinogen upon coagulation time (cp. Table IV).

Observation	Concentrations used for coagulation		Coagulation time ( <i>t</i> ) in min.	
	Plasma ( <i>z</i> ) p.c.	Venom ( <i>y</i> )	Observed	Calculated for $t = 5.15/z^{0.5}$
1	88	1 in 640,000	5.00	5.5
2	44	" "	9.00	7.8
3	22	" "	11.00	11.0
4	11	" "	14.75	15.6

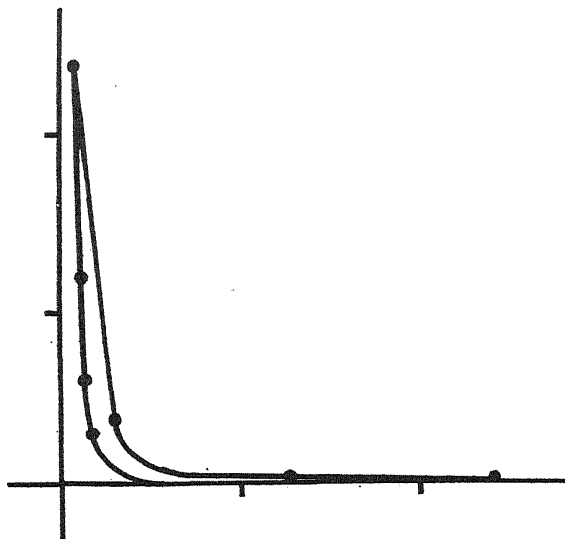


Fig. 1. The effect upon coagulation time of dilution of fibrinogen is shown in the curve to the left (Table I) and of thrombin to the right (Table IV). Scale: abscissæ, periods of 100 min.; ordinates, concentration of plasma, 39 p.c. (curve to left), or venom, 1 in 1,540,000 (curve to right). Obs. 1-7, Tables I and IV, form a single series.

left in Fig. 1. In this and other experiments the relation between coagulation time (*t*) and relative concentration of fibrinogen, *i.e.* percentage of plasma (*z*), is given by the equation

$$t = \frac{a}{z^p}, \quad \dots\dots(1)$$

where *a* is a constant varying in individual experiments and having the value 5.15 for that given in Table I. The constant *p* lies within a limited range of values: its average value in numerous experiments was 0.5.

Relative values of the content of fibrinogen in different solutions of fibrinogen may be readily obtained by equation (1) from the coagulation

times observed when the same amount of thrombin has been added to each solution. If the concentration of fibrinogen is very low some difficulty may, however, be experienced in ascertaining the exact moment at which fibrils first appear, the thickness of the fibrils becoming exceedingly small so as to render rapid recognition impossible. In a dilution containing 0.75 p.c. of normal human plasma fibrils cease to appear and "setting" is difficult to detect or no longer recognizable (Table II). The value of  $p$ , it may be noted, was found to range in different experiments between 0.35 and 0.60.

Another method of estimation which avoids the difficulty of exact determination of coagulation time in high dilutions of fibrinogen, when fibrils are of liminal visibility, is the following, based upon ascertainment of the point at which visible fibril formation ceases. A series of dilutions of the liquid whose content of fibrinogen is to be determined is made, and to each the same amount of thrombin is added, a sample of each being then placed in a glass cell, sealed with vaseline, for examination under

TABLE II. The dilution of plasma at which addition of thrombin fails to give rise to visible fibrils of fibrin.

Observation	Concentrations used for coagulation		Condition of clot		Description of plasma	
	Plasma p.c.	Venom	Consistence	Fibrils	Sample	Age (days)
1	0.75	1 in 320,000	Very soft	Absent	<i>a</i>	0
2	"	1 in 1,280,000	Soft	"	<i>b</i>	0
3	"	1 in 320,000	Very soft	"	<i>c</i>	1
4	"	1 in 1,280,000	Remained liquid	"	<i>d</i>	1
5	"	1 in 320,000	Soft	"	<i>a</i>	7
6	"	1 in 5,120,000	Very soft	"	<i>b</i>	43

dark-ground illumination at the end of 24 hours. The dilution at which visible fibrils cease to be formed is in this way ascertained. By a comparison of determinations thus made for different solutions, an estimate of the corresponding relative concentrations of fibrinogen is obtained. It will be noted that, in this method, only the presence or absence of fibrils is noted, no attempt being made to determine the time at which fibrils become visible. The dilution at which visible fibril formation ceases corresponds, as already mentioned, to a concentration of normal human plasma of 0.75 p.c.

By the use of these two methods relative estimations of fibrinogen may be made. In order to obtain absolute determinations a standard value of fibrinogen is required. This cannot be given in terms of a definite amount of fibrinogen owing to the circumstance that it is not at present



possible to obtain fibrinogen in a state of purity or even in a stable form, but a natural unit of concentration is furnished by human blood plasma, which in health appears to exhibit very small variation of its content of fibrinogen. The limited range of variation is shown by the appearance of nearly identical coagulation times when simultaneous determinations are

TABLE III. Experiment illustrating the extent of variation of content of fibrinogen in human plasma.

Observation	Concentrations used for coagulation		Coagulation time (min.)	Source of plasma
	Plasma p.c.	Venom		
1	47	1 in 1,767,000	15.0	A
2	"	" "	15.0	B
3	"	1 in 2,580,000	19.5	A
4	"	" "	19.5	B
5	"	1 in 394,000	5.3	A (1)
6	"	" "	5.3	A (14)
7	"	" "	5.2	A (66)
8	"	" "	5.3	A (98)
9	"	" "	5.5	A (182)
10	"	" "	5.2	A (198)

made with citrated plasma from different individuals, equal amounts of thrombin being added in each case. The same is indicated still more strikingly when comparative tests are made with citrated plasma obtained at different times from the same individual, such determinations being rendered possible by the use of venom, deterioration of which at 0° C. is very slow [Barratt, 1932]. Illustrative observations are shown in Table III. In the first four, two samples of citrated plasma obtained from different individuals are found to have identical coagulation times when tested shortly after collection (1 and 2) and again 2 days later (3 and 4). The next six observations (5–10) are made with samples of human plasma from the same subject, taken at intervals (given by the figures in brackets in column 5) during a period of 198 days. Since the coagulation time is practically identical in these experiments, it follows that neither the content of thrombin in the solution of venom employed, which was made up two years before the first experiment, nor the content of fibrinogen in the samples of plasma, varied in any marked degree during the period of experiment. The plasma in Obs. 5–10, it may be observed, was examined shortly after collection of blood except in Obs. 10, in which it was 24 hours old when used. The content of fibrinogen in normal human plasma thus furnishes a standard, which may be taken to represent unit concentration. Estimations in terms of this unit may be made in liquids containing fibrinogen by either of the two methods given above.

*Estimation of thrombin.*

If thrombin is added to plasma in diminishing amounts, coagulation takes place with increasing slowness. The relation between the time required for "setting" to occur ( $t$ ) and the amount of thrombin added ( $y$ ) was found by Fuld [1902] to be given by the equation

$$\log \frac{t_1}{t_2} = 0.585 \log \frac{y_2}{y_1}. \quad \text{.....(2)}$$

Martin [1905], using snake venom, observed the relation

$$ty = \text{constant} \quad \text{.....(3)}$$

when the quantity of thrombin added was small: with large amounts of thrombin the observed values of  $t$  were greater than the calculated periods. Rettger [1909] also noted that equation (3) failed if a maximum amount of thrombin was employed, further increase of thrombin no longer causing a diminution of the time of coagulation. Similar results were obtained by Barratt [1915].

If, however, the plasma employed is citrated and the coagulation period taken as the time required for the formation of visible fibrils of fibrin (this being capable of more exact measurement than the occurrence of "setting") the relation

$$t = \frac{b}{y^q} \quad \text{.....(4)}$$

is obtained, where  $b$  is constant only for individual experiments, while  $q$  varies within narrow limits, ranging in different experiments between 0.65 and 0.75. This is illustrated by the experiment detailed in Table IV

TABLE IV. The effect of dilution of thrombin upon coagulation time (cp. Table I).

Observation	Concentrations used for coagulation		Coagulation time ( $t$ ) in min.	
	Plasma (z) p.c.	Venom ( $y$ )	Observed	Calculated for $q = 0.000475/y^{0.7}$
1	88	1 in 640,000	5	5.5
5	"	1 in 5,760,000	28	25.5
6	"	1 in 51,840,000	128	119.0
7	"	1 in 155,520,000	246	257.0

and shown in the curve to the right in Fig. 1. In this experiment the constant  $b$  has the value 0.000475, and the exponent  $q$  is taken at 0.7, the coagulation times calculated in the fifth column agreeing, it will be seen, fairly closely with the observed periods. Experiments of this type afford a valuable method of making comparative estimations of thrombin in venom dilutions.

Absolute estimations may also be made. These are based upon the relation observed between the amounts of fibrinogen and thrombin taking part in the production of fibrin. If thrombin is added to citrated plasma it is usually found that the fluid expressed from the resulting clot at the end of several days, when the interaction between fibrinogen and thrombin may be regarded as complete, contains thrombin if a relatively large amount of thrombin has been added, while, on the other hand, fibrinogen is present if only a relatively small amount of thrombin is used. This is shown by the addition of citrated plasma to the expressed fluid in the first case, and of venom in the second, when clotting occurs. If, however, fibrinogen and thrombin are mixed in suitable proportions, the expressed fluid is found to be free from either, that is to say, the further addition of plasma or thrombin no longer causes clotting. This is illustrated in Table V: in Obs. 1, 2 and 3 fibrinogen is absent from the expressed fluid,

TABLE V. Determination of the amount of thrombin required to convert fibrinogen into fibrin.

Observation	Concentrations used for coagulation		Approximate ratio of fibrinogen to thrombin	Content of fluid expressed from clot	
	Plasma p.c.	Venom		Fibrinogen	Thrombin
1	47	1 in 21,500,000	50,000 : 1	—	+
2	70	1 in 41,000,000	143,000 : 1	—	+
3	80	1 in 71,000,000	284,000 : 1	—	+
4	80	1 in 144,000,000	576,000 : 1	+	—
5	10	1 in 144,000,000	72,000 : 1	—	+
6	86	1 in 331,000,000	143,000 : 1	—	+
7	10	1 in 568,000,000	284,000 : 1	—	+
8	10	1 in 1,135,000,000	576,000 : 1	+	—

while in Obs. 4 it is present, the converse being true of thrombin. If it is assumed that fibrinogen and thrombin are in corresponding amounts midway between Obs. 3 and 4, the relation between fibrinogen and thrombin would be in the neighbourhood of 384,000 : 1, the plasma employed being assumed to contain 0.5 p.c. of fibrinogen and venom being regarded as pure thrombin. This proportionality, which does not seem to be altered by dilution—in Obs. 5–8, in which the concentrations used are about one-eighth of those in Obs. 1–4, a similar relation is indicated—renders possible the estimation of thrombin in terms of a unit, for it is obvious that if normal human plasma is taken as representing unit concentration of fibrinogen, then the concentration of thrombin just sufficient to convert all the fibrinogen present in the plasma into fibrin must be taken to represent unit concentration of thrombin. It is, however, preferable to indicate this concentration by the corresponding coagulation time rather

than to attempt to express it in terms of thrombin. Experiments made to determine this period—that is to say, the coagulation time when thrombin is added in amount just sufficient to convert all the fibrinogen present in undiluted normal human plasma into fibrin—were found to give a mean value of approximately 135 min., corresponding to a 1 in 77,500,000 concentration of the sample of venom used in Obs. 1–7, Tables I and IV.

Using this value of  $t$  for unit concentration, the following illustration of the determination of titre of a solution of thrombin may be given. In Obs. 1, Table I, the observed value of  $t$  for 88 p.c. plasma is 5 min. The value of  $t$  for undiluted plasma is by equation 1, p. 423,

$$5 \times \left( \frac{0.88}{1.00} \right)^{0.5} = 4.7 \text{ min.}$$

The concentration of thrombin employed in Table I is, therefore, by equation 4, p. 426,  $\left( \frac{135}{4.7} \right)^{1.43} = 121$  times unit concentration.

#### SUMMARY.

1. Methods of estimation of fibrinogen and thrombin, based upon the observed effect of dilution, are described.

2. As units the following are employed:

(a) Unit concentration of fibrinogen: the concentration present in normal human plasma.

(b) Unit concentration of thrombin: the concentration which is just sufficient to convert all the fibrinogen present in normal human plasma into fibrin.

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## THE VASO-DILATOR ACTION OF ADRENALINE.

By G. A. CLARK.

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It is now generally recognized that very small doses of adrenaline given intravenously, especially in certain conditions of anæsthesia [Macdonald and Schlapp, 1926; Vincent and Curtis, 1927], can produce a fall in blood-pressure in the cat or in the dog. It has also been shown that these minute amounts of adrenaline cause vaso-dilatation which apparently has its site in the capillary field [Dale and Richards, 1918]. Earlier work by Cannon and Lyman [1913] indicated that this peripheral dilatation was not limited to any particular region or tissue of the body, for they were able to obtain a depressor effect with adrenaline after clipping carotid, subclavian and iliac arteries, and the fall in pressure was not much less than that seen when these arteries were open and the superior and inferior mesenteric, coeliac axis and renal arteries were clamped. Experiments by Gruber [1929], however, on unanæsthetized animals showed that small doses of adrenaline regularly caused dilatation in the vessels of voluntary muscle, judged by the increased venous out-flow, but that not in every case did the general blood-pressure fall; this the author interpreted as indicating that adrenaline dilated muscle vessels but, in those cases where the pressure remained constant, constricted vessels in other regions of the body. There is much evidence to show that the action of adrenaline varies both qualitatively and quantitatively in different parts of the body; thus, the coronary vessels are dilated [Hammouda and Kinosita, 1926], the vessels of voluntary muscle are also dilated [Hoskins, Gunning and Berry, 1916; Hartman and McPhedran, 1917; Gruber, 1929; Clark, 1930] except where large doses are given [Erlanger and Gasser, 1919; Gunning, 1917; Gruber, 1929], while skin vessels appear to be uniformly constricted [Hoskins, Gunning and Berry, 1916; Clark, 1930]; the splanchnic vessels also respond by constriction, but, if the adrenaline given raises

the general blood-pressure, constriction is changed to dilatation which is partly reflex and partly passive [Clark, 1930]. The vaso-dilatation caused by adrenaline in skeletal muscle is a purely peripheral effect, for it has been seen after cutting all nerves to a limb [Dale and Richards, 1918] and also in the isolated perfused limb [Dale and Richards, 1927], but there is no conclusive evidence to show whether or not adrenaline in small doses can exert on all minute vessels in the body a dilator action which is definitely peripheral.

It seemed that a reinvestigation of the problem was necessary to determine whether or not vessels in the skin and in the splanchnic area would respond as do those in skeletal muscle, and, if possible, to determine under what conditions dilatation or constriction predominates.

Cats under chloralose anaesthesia were used and vascular changes were determined by recording the venous outflow from muscle, skin or intestine, clotting being prevented by intravenous injection of "Novirudin." After falling on an electric drop recorder, the blood was collected in a beaker kept at 39° C. by means of a water jacket and was returned to the animal by a burette connected with one external jugular vein. In the case of muscle a cannula was inserted into the femoral vein and, to ensure that the flow came only from muscle, the leg was tightly ligated immediately above the ankle and all cutaneous veins tied; in some cases the leg was skinned and the skin replaced with the help of sutures so that all chance of anastomosis between skin and muscle was prevented.

Blood flow from the skin was observed by recording the outflow from the superficial femoral vein; special care was taken to keep the vein in the vicinity of the cannula moist and covered by skin to prevent contraction.

Intra-arterial injection of adrenaline was made by inserting the needle of a suitable syringe into the stump of the common origin of the internal iliac arteries, after tying off all branches; the needle could be pushed up till the point was in the stream of the external iliac. Repeated injections were given by leaving the syringe *in situ*, taking precautions to prevent blood entering and pushing up the piston.

In the case of the mesenteric vessels a loop of intestine was isolated from the rest of the gut and a cannula inserted into the vein draining the loop. Intra-arterial injections were given through the stump of a branch of the artery supplying the loop. It sometimes happened that the blood flow through the intestine was irregular due to contractions of the intestinal wall, but this could be prevented by intravenous injection of atropine which appeared in no way to interfere with the action of adrenaline to be described below.

Special care was taken to maintain the temperature of the cat at  $39.2^{\circ}\text{C}$ . which is the mean normal found by Lee and Scott [1914], because Rein [1930] is of the opinion that adrenaline dilates muscle vessels only when the body temperature is subnormal or when the muscle is active. A few experiments were made at temperatures above and below the normal.

*Vessels of voluntary muscle.* The sequence of events following the intra-arterial injection of a minute dose of adrenaline is shown in Fig. 1.

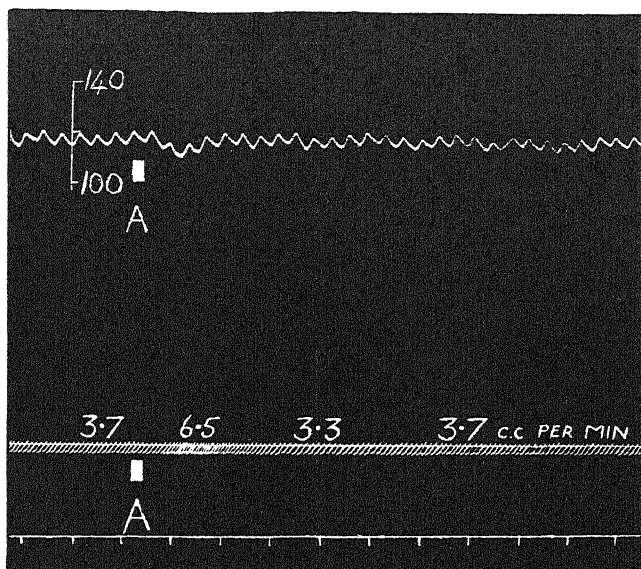


Fig. 1. Blood-pressure and venous outflow from muscles of hind leg. Skin of leg separated from underlying tissues and held in place by stitches. Temp.  $39.2^{\circ}\text{C}$ . Time in 10 sec. At A, 0.05 c.c. adrenaline  $1 \times 10^{-6}$  into iliac artery.

There is a slightly shorter latent period in the blood-pressure record than in that of the blood flow from the muscle, which is to be expected because of the time which must elapse before the increased flow reaches the cannula; following the latent period and during the slight fall in blood-pressure the venous flow is increased for a time and then decreased before it returns to its original rate. These changes are almost invariably seen after giving adrenaline as described; they appear to be independent of any temperature changes likely to be found under physiological conditions, for Fig. 2 was obtained from a cat whose rectal temperature had

been allowed to fall to  $36.0^{\circ}\text{C}$ ., i.e.  $3.2^{\circ}\text{C}$ . below the mean normal temperature, while a similar response was seen at  $41.3^{\circ}\text{C}$ .

Adrenaline dilatation has also been seen in limbs denervated from 5 min. to 2 hours before experiments where no sign of muscular activity was evident. These results, therefore, do not confirm those of Rein [1930] already referred to.

An effect, similar to that shown in Fig. 1, has been obtained by injecting intra-arterially 0.1 c.c. of blood from the suprarenal vein of a second cat under chloralose anæsthesia during stimulation of the splan-

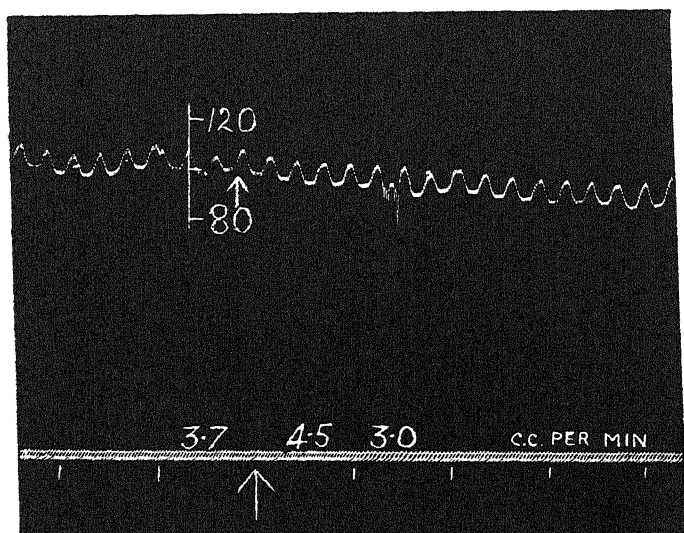


Fig. 2. Blood-pressure and muscle blood flow. At  $\uparrow$  0.05 c.c. adrenaline  $1 \times 10^{-6}$  into iliac artery. Temp.  $36.0^{\circ}\text{C}$ . Time in  $\frac{1}{2}$  min.

nic nerve; injection of the same amount of blood from the inferior vena cava before splanchnic stimulation gave no dilatation.

By giving repeated doses of adrenaline at short intervals the constrictor effect of one injection can be eliminated or cut short by a subsequent injection (Fig. 3). Where the time of injection is prolonged as in Fig. 4 B, it will be seen that no definite constriction occurs during the injection; in fact, the rate of flow is actually increased, and this cannot be attributed to the volume of fluid injected, as 0.1 c.c. is equivalent to less than two drops of the size here recorded. In Fig. 3 the same amount of adrenaline was given, not continuously as in Fig. 4 B, but with a pause



between the five fractions into which the dose was divided. The dilator effects of the first two fractions are combined and the third appears to

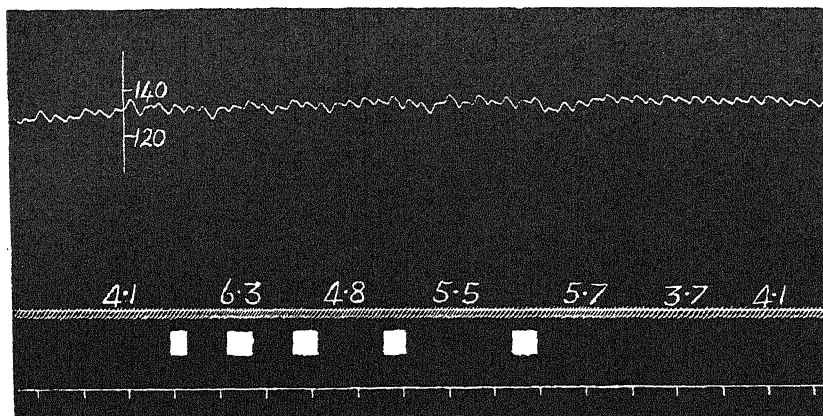


Fig. 3. Blood-pressure and muscle blood flow. At each white area 0.03 c.c. adrenaline  $1 \times 10^{-6}$  into iliac artery. Figures above the drop record indicate flow in c.c. per min.

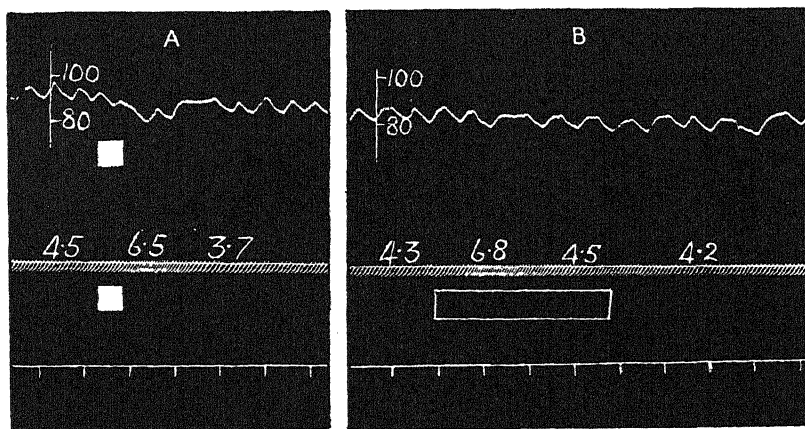


Fig. 4. Blood-pressure and muscle blood flow. A. 0.10 c.c. adrenaline  $1 \times 10^{-6}$  at white area. B. 0.15 c.c. adrenaline  $1 \times 10^{-6}$  at enclosed area. Time in 10 sec. Figures above the drop record indicate flow in c.c. per min.

have less effect than any of the others although it definitely cuts short the constrictor phase judged by the duration of this latter after the third, fourth and fifth fractions. The dilatation following the fourth fraction is

slightly less than that following the fifth, and these differences may be related to the progressively longer intervals between the beginning of any two injections.

The suggestion of Cannon and Lyman [1913] that the production of dilatation or constriction by adrenaline depends on whether the vessels are initially constricted or dilated is not borne out by experiments in which repeated small amounts of adrenaline are given so that the dilator phase is prolonged; the first two injections in Fig. 3 give an example of this. Nor is their point of view upheld by the fact that dilatation may be seen when the blood pressure is definitely below the "critical level" given by them (Fig. 5).

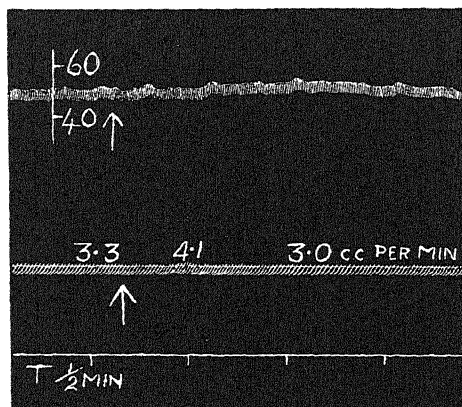


Fig. 5. Blood-pressure and muscle blood flow. At  $\uparrow$  0.05 c.c. adrenaline  $1 \times 10^{-6}$  into iliac artery.

Although the phenomena described above are those usually seen as a result of giving small doses of adrenaline into an artery, exceptions have been observed in which only a constriction and no dilator phase occurs with the smallest amount of adrenaline which gives a response. Other exceptions, still rarer, are those where only constriction is seen with a very small dose, but a brief dilatation occurs in the response to a larger dose. These apparently anomalous cases require further investigation.

*Intestinal vessels.* The effect of small intra-arterial injections of adrenaline on the blood flow through the intestinal vessels is shown in Fig. 6. The smallest dose which has any detectable effect always causes vaso-constriction, judged by the diminished venous outflow. It might be objected that the result here shown would be more likely to occur in gut,

the vessels of which had lost their tone due to the necessary operative manipulations, but in all experiments the intestine was handled as little

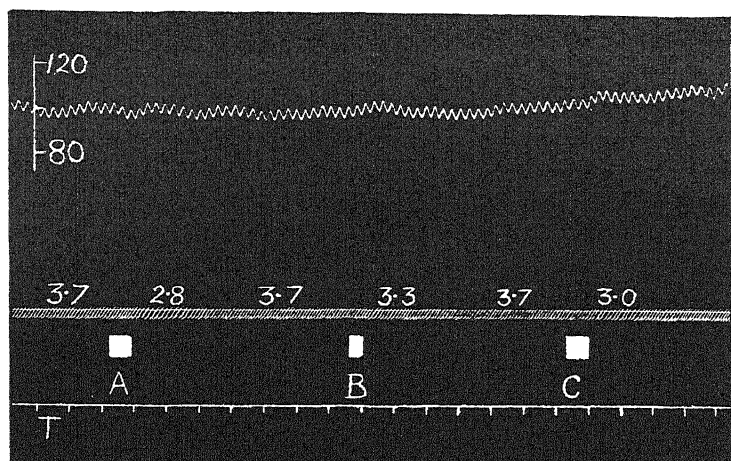


Fig. 6. Blood-pressure and venous flow from loop of intestine. At A, 0.05 c.c. adrenaline  $1 \times 10^{-6}$  intra-arterially. At B, 0.01 c.c. adrenaline  $1 \times 10^{-6}$  intra-arterially. At C, 0.02 c.c. adrenaline  $1 \times 10^{-6}$  intra-arterially. Time in 10 sec. The figures above the drop record indicate flow in c.c. per min.

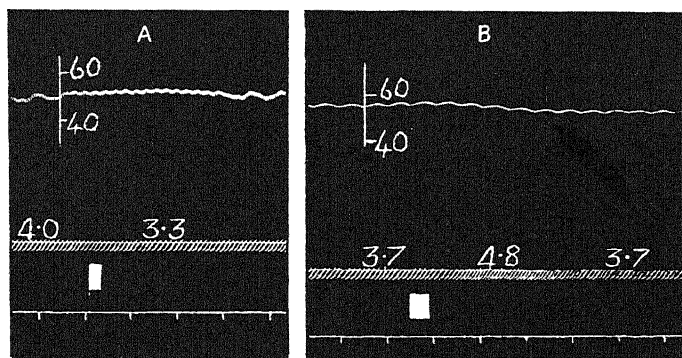


Fig. 7. Blood-pressure and venous flow from loop of intestine. Between A and B, 0.6 mg. ergotamine per kg. intravenously. A. 0.01 c.c. adrenaline  $1 \times 10^{-6}$  intra-arterially. B. 0.10 c.c. adrenaline  $1 \times 10^{-6}$  intra-arterially. Time in 10 sec. The figures above the drop record indicate flow in c.c. per min.

as possible and was always left in the abdominal cavity covered by omentum. The blood-pressure does not suggest any degree of shock and, moreover, the same response to adrenaline shown in Fig. 6 can be obtained

during the rise in blood-pressure caused by an intravenous injection of posterior pituitary extract. It seems therefore that the minute vessels of the intestine respond to the direct action of adrenaline in a manner qualitatively different from that of the vessels in voluntary muscle.

Dale [1913] has shown that stimulation of the splanchnic nerve after intravenous injection of ergotoxine will cause a fall in blood-pressure and interprets this as evidence of the presence of vaso-dilator fibres in this nerve. Fig. 7 shows that intra-arterial adrenaline will produce dilatation in the mesenteric vessels if ergotoxine has been previously given, and here again the initial pressure is below the "critical level" of Cannon and Lyman [1913]. It is apparent that dilatation is still possible with a general blood-pressure below 60 mm. Hg in Fig. 7 B, and therefore its failure to appear in Fig. 7 A is not due to initial maximal dilatation.

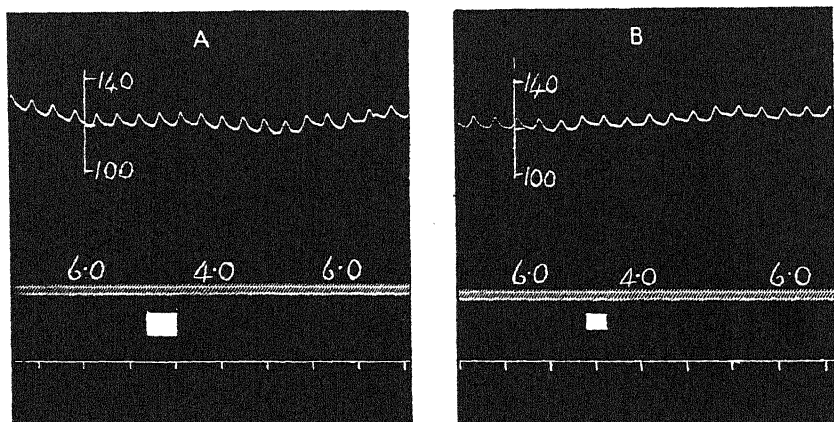


Fig. 8. Blood-pressure and venous flow from skin of hind leg. A. 0.02 c.c. adrenaline  $1 \times 10^{-6}$  into iliac artery. B. 0.05 c.c. adrenaline  $1 \times 10^{-7}$  into iliac artery. The figures above the drop record indicate flow in c.c. per min.

*Skin vessels.* The minute vessels of the skin appear to respond directly to very small doses of adrenaline in the same way as do those of the intestine, but previous work in which plethysmographic methods were used indicates that the constriction in skin is more intense, in that it does not give way to dilatation when the blood-pressure rises [Clark, 1930]. In the case of skin, however, it is difficult, when recording changes of flow only, to determine to what extent these changes are due to alterations in calibre of the arterio-venous anastomoses described by Grant and

Bland [1931] and to what extent they are due to variations in capillary calibre. Taking into consideration previous investigations by direct observation [Hoskins, Gunning and Berry, 1916; Lewis, 1927] and by plethysmographic methods [Clark, 1930], however, the conclusion may be drawn that adrenaline in amounts which have any detectable action causes constriction of the minute skin vessels. Fig. 8 shows the effect on venous outflow from the skin of the hind leg of the intra-arterial injection of adrenaline. In no case was an increased flow found.

If sympathetic vaso-dilator fibres to the vessels of the skin exist, it should be possible to unmask them by injecting adrenaline after giving ergotoxine. In several experiments in which this was done there was no increased rate of flow through the skin vessels; the dose of ergotoxine given was 2.5 mg. per kg., and following this 0.05 c.c. of adrenaline diluted 1 in 1 million injected intra-arterially gave a transient fall in blood-pressure varying from 5 to 10 mm. Hg. The ergotoxine caused considerable slowing of the blood flow through the skin.

*Sympathin.* Since the greater part of the experimental work described above was done, Cannon and Rosenblueth [1933] have produced direct evidence for the presence of a substance, Sympathin E, which is released by stimulation at vaso-constrictor sympathetic nerve endings. Evidence is also given of a similar substance, Sympathin I, formed by dilator or inhibitor nerve endings. These findings amply explain the dual effect in the vessels of muscle of an intra-arterial injection of adrenaline which presumably releases both Sympathin E and Sympathin I; it seems, however, that the dilator endings have a lower threshold to adrenaline than do the constrictor.

Experiments were devised to determine the possibility of showing the presence of Sympathin I. Two cats were anaesthetized with chloralose and one of these (*A*) was prepared as previously described to record the blood flow from a group of muscles in the hind leg. In the second cat (*B*) the suprarenal glands were tied off with a pedicle ligature and a portion of the portal vein laid bare; the blood-pressure was recorded in the usual way. Ergotoxine was then given (2.5 mg. per kg.), and when the blood-pressure had settled at its new level adrenaline was injected. Samples of blood were taken from the portal vein (*a*) before giving adrenaline, and (*b*) at various intervals after adrenaline; 0.1 c.c. of these samples was injected into the iliac artery of cat *A*. It was found that the samples (*a*) had no effect on muscle blood flow and the samples (*b*) taken within 10 min. of giving adrenaline were also usually without effect, but samples taken between 10 and 15 min. gave a dilator response (Fig. 9); if, however, the

adrenaline had caused a rise in blood-pressure in cat *B* this dilator response was followed by constriction (Fig. 10). A simple calculation and the lack of response to earlier samples (*b*) showed that the amount of adrenaline given to cat *B* was not responsible for this effect, and the conclusion is that a dilator substance had been released from sympathetic inhibitor and dilator nerve endings in cat *B*.

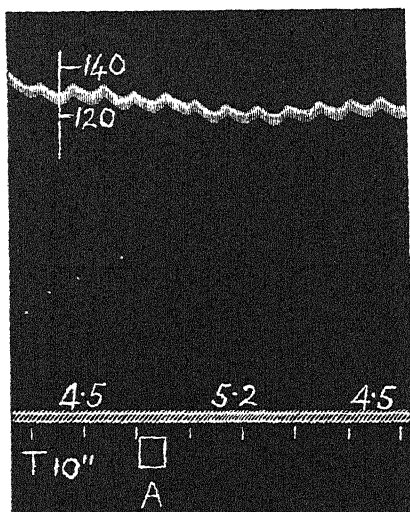


Fig. 9.

Fig. 9. Blood-pressure and venous flow from muscle of hindlimb. At A, 0.1 c.c. blood intra-arterially, from second cat given 0.4 c.c. adrenaline ( $1 \times 10^{-4}$ ) intravenously after ergotoxine; blood drawn from portal vein 13 min. after adrenaline given. The figures above the drop record indicate flow in c.c. per min.

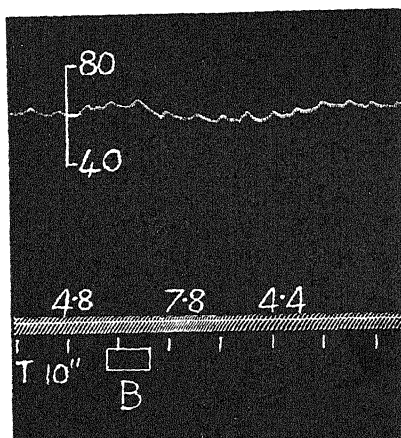


Fig. 10.

Fig. 10. Similar to Fig. 9, but adrenaline in second cat had caused a rise in general blood-pressure despite ergotoxine. The figures above the drop record indicate flow in c.c. per min.

### DISCUSSION.

The double action of adrenaline, dilator and constrictor, has been discussed by Dale and Richards [1918] and is well shown as the result of a single injection in the volume record of a skinned limb in Fig. 15 D of their paper; the fact there shown that the volume of the muscle alters in a manner similar to the venous outflow clearly indicates that the increased outflow is not due to constriction of vessels squeezing more blood out of the muscle but is a true expression of vaso-dilatation. Dale and Richards are of the opinion that both dilatation and constriction have their site in the capillaries, and this view is supported to some extent

by the result described above of giving repeated injections of adrenaline (Fig. 3); if in this case dilatation took place in the capillaries but constriction in the arterioles a second injection during the action of the first would merely intensify the double effect, and further arteriolar constriction, in the absence of a rise in blood-pressure, would prevent any increase in venous outflow. Again, if we assume, contrary to the evidence of Dale and Richards, that small doses of adrenaline dilate arterioles and constrict capillaries simultaneously, it is highly improbable that a second injection by increasing arteriolar dilatation (and presumably further constricting capillaries) would cut short the constrictor effect in the capillaries by mere passive dilatation in view of the pressures which Lewis [1927] has shown constricted capillaries can withstand. It is true that Lewis's observations refer to the minute vessels in human skin and therefore may not be directly applicable to capillaries in muscle, but in the latter vessels adrenaline dilatation has been obtained when the arterial pressure was as low as 50 mm. Hg (Fig. 5), a pressure against which Lewis has shown that even dilated capillaries can constrict. The fact that vaso-dilatation due to adrenaline occurs at this low pressure does not support the view of Cannon and Lyman [1913] that the effect of adrenaline depends on the state of the vessels in any given case.

It does not seem possible in the present state of knowledge to harmonize the observation that repeated small injections of adrenaline produce prolonged or repeated dilatation (Figs. 3 and 4 B) with that of Dale and Richards [1918] and of Burn [1932] that a low concentration of adrenaline added to blood perfusing a limb will restore constrictor tone in the minute vessels. Some experiments as yet incomplete, however, suggest that an explanation of the apparent discrepancy may be found, but the problem must be left over for further investigation.

The difference in reaction between intestinal vessels and the vessels in muscle cannot be due to the absence in the former of the means for active dilatation, for this latter reaction can be brought about by adrenaline after ergotoxine; there is evidence that sympathetic vaso-dilator fibres are relatively few in the splanchnic area [Cannon and Rosenblueth, 1933], and it may be that their threshold to adrenaline is also relatively higher than in muscle. In the skin it does not seem possible to show the presence of sympathetic dilator fibres by the technique adopted here; conditions are probably complicated by the presence of arterio-venous anastomoses [Grant and Bland, 1931], and it is impossible to tell by means of blood-flow methods whether changes in flow caused by adrenaline are due to variations in calibre of these anastomoses or of capillaries. It does appear, however, that adrenaline, in the smallest

amount which has any effect, always causes a diminution in blood flow through the skin and that this response is not reversed by previous administration of ergotoxine.

#### SUMMARY AND CONCLUSIONS.

The action of minute amounts of adrenaline given intra-arterially on the blood flow through skeletal muscle, intestine and skin in the cat has been investigated.

The response of muscle vessels to a single injection is of a twofold nature, first dilatation and then constriction, although cases are recorded in which only constriction was seen with the smallest dose of adrenaline that would give any response. This response to adrenaline in the vessels of skeletal muscle is not qualitatively altered by any change in body temperature likely to be found under physiological conditions. Evidence is given of the release of a dilator substance analogous to Cannon's Sympathin I when only inhibitor or dilator sympathetic endings are stimulated by adrenaline, stimulation of constrictor endings being prevented by ergotoxine.

The smallest effective amount of adrenaline always produces a diminished flow of blood through the intestine and skin. In the intestine, but not in the skin, this response is reversed by previous injection of ergotoxine.

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## "INHIBITION" IN MEDULLATED NERVE.

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FENG and Hill [1933] have recently studied the heat production of frog's nerve during the prolonged application of a stimulus of known frequency. They have shown that with a relatively low frequency (50–100 shocks/sec.), after a certain time a constant rate of production of heat is arrived at; this can be maintained for a very long time but diminishes, rapidly at first and then gradually, when excitation ceases, finally returning to the level corresponding to rest. With a high frequency, however (500–1000 shocks/sec.), a steady production of heat cannot be obtained; the rate at which heat is produced increases rapidly at the beginning of excitation, reaches a maximum, then decreases in spite of the maintenance of the stimulus. If, during this state of apparent fatigue, a stimulus of high frequency is replaced by one of low frequency, an immediate increase is observed in the rate of production of heat: the nerve, which no longer responds to the excitation of high frequency, is nevertheless capable of responding to an excitation of low frequency. This phenomenon, which was further discussed by Hill at the 1933 meeting of the American Association in Chicago [1934], is the subject of the present investigation.

### TECHNIQUE.

The apparatus used was similar to that described by Hill [1932] and by Feng and Hill [1933]. The thermostat, however, had been modified as follows. The wooden box, which forms its external cover, instead of being placed directly in the room, was enclosed in a second wooden box covered inside with zinc sheet. The air space, separating the two boxes, was maintained at a constant temperature by an electrical heating arrangement automatically regulated. The temperature of the air space

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could easily be maintained constant within  $0.1^{\circ}\text{C}$ . This made unnecessary the maintenance of a constant temperature in the room as a whole, and increased considerably both the thermal stability and the ease of working.

The deflection of the galvanometer was read directly on a graduated scale without photographic recording. The scale was placed 4.5 metres from the galvanometer and the spot could easily be read to the nearest millimetre. Readings were made every 15 sec. during the first few minutes following the application or removal of a stimulus, and every minute later on, when an approximately steady state had been reached.

As stimuli, condenser discharges were employed at a frequency determined by the speed of rotation of a commutator [Hill, 1932]. Since the frequency had to be varied abruptly, two commutators were used running at different speeds. The reversal of the key allowed an instant transfer from one frequency to the other. One of the commutators was used for low frequencies (50–100 shocks/sec.), the other for higher frequencies (usually 500–1000 shocks/sec.). The condenser discharges themselves were exactly the same whatever the frequency, since they depended only upon the capacity of the condenser, which was the same in every case, and the resistance through which it discharged, which was also the same.

Frogs' sciatics were used, from large Hungarian *R. esculenta*, eight nerves in each experiment.

In general, in order to avoid polarization at the stimulating electrodes, excitation was effected by alternating charge and discharge of the condenser; thus excitation occurred alternately at one electrode and the other. In certain experiments, however, condenser discharges alone were employed, the stimuli being all in one direction, the cathode being at the electrode which was nearer to the thermopile; in certain other experiments there were two pairs of electrodes, each pair being used for one of the two frequencies. A suitable manipulation of the keys allowed an instant transfer from one mode of stimulation to the other (alternating or "one-way") and the use, at will, of one or other pair of electrodes. Actually three electrodes only were necessary, *A*, *B* and *C*, in that order pointing towards the thermopile. The distances were: *A* to *B*, 3 mm.; *B* to *C*, 12–15 mm.; *C* to thermopile, 18 mm. The distance from *B* to *C* was so great that stimulation at *A* or *B* could scarcely have affected the nerve at *C*. For the lower frequencies *A* and *B* might be employed, *B* being the cathode: for the higher frequencies, *B* and *C*, *C* being the cathode. *C* was actually the block of silver ordinarily serving to prevent the passage of heat from the stimulating electrodes to the thermopile [see Hill, 1932,

p. 113, Fig. 3]. The usual electrodes *A* and *B* will be described as the "upper" electrodes.

The capacity of the condenser used for stimulating, the potential to which it was charged, and the resistance short-circuiting the nerve, were chosen according to the principles discussed by Hill [1932]. In view of the high frequency of excitation employed, it was necessary to ensure that the condenser discharges were complete in the short interval of contact on the commutator. Unless this precaution be taken the shocks making up the stimuli will not be independent of the frequency. The capacity required can be determined by calculation, assuming for example that not more than 1 p.c. of the energy of the condenser should be undischarged at the moment when the discharge is cut short.

The following test was made. In the ordinary excitation circuit, namely, nerve in series with 5000 ohms, short-circuited by 1000 ohms, the latter was replaced by a resistance wire (932 ohms) wound non-inductively on a thermopile. The system could be connected to one or other of the commutators. The deflection of a galvanometer connected to the thermopile on which the hot wire was wound allowed one to measure the energy of the current through the wire. With a known frequency of charge and discharge one could determine the maximum capacity which would allow discharge to be complete to any required degree in the time available between separate contacts. Provided that the contacts are clean and not too oily, and the brush in good order, the rules are obeyed.

With a capacity of  $0.105\mu\text{F}$  the readings obtained with the commutator and those with a constant current calculated to give the same energy were compared. Between 100 and 1200/sec. the difference was never more than 4 p.c. At a frequency of 1950 the difference had risen to 12.5 p.c. In the experiments reported the stimuli were always as follows: capacity,  $0.105\mu\text{F}$ , charged to 14.4 volts, 5000 ohms in series with nerve, nerve and resistance short-circuited through 1000 ohms: time of half discharge,  $0.06\sigma$ ; of 99 p.c. discharge,  $0.4\sigma$ . These shocks were well super-maximal. In general the frequency was kept below 1200/sec.

In order to keep permanent control during each experiment on the effectiveness of the stimulus, the following arrangement was finally adopted. The 1000 ohms shunt ordinarily used was replaced by a hot wire wound on a thermopile, the resistance of the hot wire being 932 ohms. At any point during an experiment a reading of a microammeter connected to the thermopile on which the hot wire was wound, is a measure of the energy in the stimulus. During each experiment several readings were made on the microammeter; the readings were always consistent and provided evidence of the efficiency and constancy of the stimuli.

The experiments were all made at a temperature between  $21.2$  and  $22.7^\circ\text{C}$ .

### *Sensitivity.*

The apparatus was calibrated by observing the steady deflection produced by a series of regular condenser discharges applied between the heating electrodes after the nerve had been made inexcitable by prolonged

treatment with nitrogen. The sensitivity was practically constant, the following being the mean value: 1 mm. of steady deflection corresponded to  $1.81 \times 10^{-8}$  cal./sec., or to  $12.9 \times 10^{-8}$  cal./g./sec.

### *Heat leak.*

By reason of the high frequency of the stimuli used, it was feared that the heat might leak from the stimulating electrodes along the nerve into the thermopile in spite of the usual precaution of a block of silver placed between the two. In a certain number of experiments, therefore, the effect of the various stimuli employed between their usual electrodes was tested on nerves previously killed by long residence in nitrogen. In no case could one detect any important leakage of heat. The maximum effect observed corresponded to 8 p.c. of the heat found in the same experiment with the nerves stimulated alive, and even then only with a very high frequency, namely 2000/sec. In most cases the heat leak was quite negligible.

### RESULTS.

In the first experiments the upper electrodes only (*A* and *B*) were used with stimuli alternating in direction in order to avoid polarization. Each individual shock, whether the frequency was low or high, had the same energy and time relations of discharge and was chosen to be approximately optimal for a nerve at rest.

The phenomenon observed is as follows. When, by means of an excitation of long duration and of relatively low frequency (50–100/sec.), a steady state of heat production by the nerve has been established, if the frequency is raised abruptly (*e.g.* to about 1000/sec.) an abrupt diminution is observed in the production of heat, the galvanometer moving rather rapidly in the negative direction. The initial heat production is diminished, or nearly abolished, though of course the recovery heat production from previous stimulation remains and follows its normal course. The nerve appears at first to be “fatigued.” If, however, to the nerve apparently fatigued one substitutes in place of the excitation of high frequency the previous excitation of low frequency, one observes at once a production of heat at the previous higher rate and a return to the steady state characteristic of the original low-frequency excitation. The nerve, which seemed to be “fatigued” when tested by a high-frequency stimulus, is not fatigued at all when tested by a low-frequency stimulus. The same series of phenomena can be repeated many times. Some experiments have

lasted for more than 3 hours, during which a steady state of heat production has been maintained under excitation of low frequency, interrupted by abrupt falls in the rate of heat production following the application of a stimulus of high frequency (Fig. 1).

It is important, however, to note that the high-frequency stimulus produces a maximal thermal response when applied to a resting nerve. With a stimulus of given short duration to a resting nerve, the heat increases with frequency up to very high values of the latter, as is shown by the usual heat-frequency relation described by A. V. Hill and his collaborators in several previous communications.

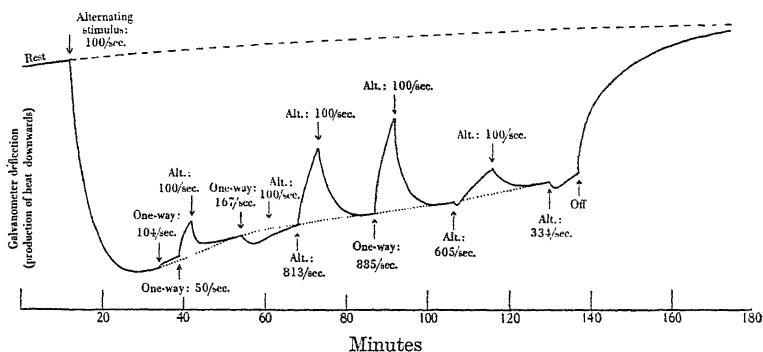


Fig. 1. Heat production of frog's nerve at about 22° C. during continuous excitation at various frequencies for 125 min. Experiment of May 15, 1933. Rate of heat production downwards as galvanometer deflection without analysis. Alternating or one-way stimuli as shown. The broken line interpolated between the beginning and the end of the experiment is a base line corresponding to the rate of heat production of the resting nerve. The dotted line corresponds to the steady and nearly constant rate of heat production during continuous stimulation at 100/sec. For further description see text.

The facts have been verified in fifteen experiments. One may begin by exciting the nerve during a state of rest by a high-frequency stimulus (for example, May 8, 10 and 26 respectively, 1284/sec., 920/sec., 920/sec.). There is an immediate and large positive deflection of the galvanometer corresponding to an intense production of heat by the nerve. At the end of 2 min., in place of the high-frequency stimulus effective on the resting nerve, a low-frequency stimulus is applied; the nerve continues to produce heat, though at first at a lower rate, and in the appropriate time reaches a steady state. The substitution now of the high-frequency for the low-frequency stimulus immediately breaks down the steady state

and causes not an increase but an abrupt decrease in the rate of production of heat. A return to the low-frequency stimulus in place of the high causes a gradual re-establishment of the previous steady condition.

The sudden diminution in the rate of heat production caused by substituting a high for a low frequency does not represent a complete

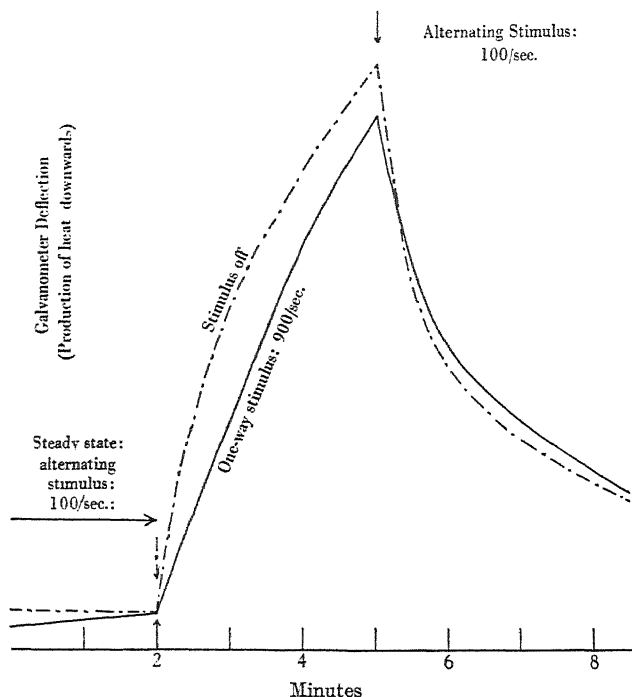


Fig. 2. Experiment of May 17, 1933. Comparison of the effect obtained during a steady state of heat production by a nerve stimulated continuously at a low frequency, 100/sec., (a) by stopping the stimulus altogether (broken line) and (b) by changing the stimulus to one of high frequency, 900/sec. Rate of heat production downwards as galvanometer deflection without analysis. Note that the high-frequency stimulus does not produce complete cessation of response.

cessation of response, at least in all the fibres. If, during the condition of diminished rate of heat production caused by applying a high-frequency stimulus as described, one stops the stimulus altogether, one immediately observes a rather more rapid fall in the rate of production of heat, as shown by an acceleration in the movement of the galvanometer towards its resting position. In one experiment (May 17) deflections in the negative

direction were compared during a steady state of stimulation at 100/sec.: (a) when excitation was stopped altogether, and (b) when a low-frequency stimulus was replaced by one at 900 shocks/sec., the interval in either case being 3 min. For complete cessation the deflection was 74 mm., for high-frequency excitation it was 67 mm. (Fig. 2). A little later in the same experiment, again for 3 min., cessation of excitation gave a negative deflection of 71 mm., a stimulus of 612 shocks/sec., a negative deflection of 55 mm. Similarly, the rise in the heat production on returning to the stimulus of low frequency is greater after an interruption in the stimulus than after an excitation at high frequency. The latter, therefore, does not cause a complete cessation of response, except perhaps at very high frequencies.

One naturally asks whether the fact that the shocks alternated in direction might not explain their inadequacy at high frequency. To test this possibility, in spite of the danger of polarization, in a certain number of experiments stimuli were employed consisting of shocks in one direction only, the electrode nearer the thermopile being made the cathode. The effect was compared with that of alternating shocks of the same frequency. First of all it was verified that with excitations of low frequency (50-167/sec.) it is a matter of indifference whether alternating or one-way stimuli are used. The steady state of heat production is not modified at all, or at most to an insignificant extent, when alternating shocks at 100/sec. are replaced by one-way shocks at the same frequency. In order, therefore, to avoid polarization alternating shocks were used at the low frequency as conditioning stimuli to obtain the steady state.

In one experiment (May 11) there were applied successively, during a steady state with an alternating stimulus at 100/sec., and replacing that stimulus, (a) an alternating stimulus of 807/sec., (b) a one-way stimulus at 884/sec., and (c) an alternating stimulus at 807/sec. The alternating stimulus gave at first in both cases a slight extra production of heat followed by a very rapid diminution. The one-way stimulus gave immediately a rapid diminution. In all three cases a return to excitation at 100/sec. gave an immediate positive deflection and a return to the steady state.

In another experiment (May 15) an alternating stimulus of 813/sec. and a one-way stimulus of 885/sec., when substituted for a steady stimulus of 100/sec., were immediately followed by a large negative deflection more rapid in the case of the one-way stimulus. A return to the stimulus at 100/sec. brought an immediate positive deflection and a return to the steady state. The same result was obtained in another experiment

(May 17). It seems therefore that high-frequency shocks in one direction produce the same phenomenon as alternating shocks of the same frequency, and that it is not the alternation in direction of the latter that is the cause of their inadequacy. Indeed, the diminution of heat is slightly greater when the shocks are in one direction only. This may be due to an effect of polarization adding on to the effect of the high frequency, but it is at least possible that with alternating shocks at electrodes *A* and *B*, when the frequency is high, the impulses set up at *A* are unable to pass electrode *B* and so to reach the thermopile. Thus, alternating shocks at 1000/sec. at *A* and *B* may effectively be no more than one-way shocks at 500/sec. at *B*. If so, the high-frequency effect would be more obvious with one-way shocks at 1000/sec. at *B*.

In one experiment (May 26) the following result was observed. During the state of diminished heat production caused by substituting a one-way stimulus of 1000/sec. for an alternating stimulus of 100/sec. during a steady state caused by the latter, an alternating stimulus of 1090/sec. was applied. The immediate effect was a very slight positive deflection followed by a negative deflection similar to that preceding it. It seems, therefore, that one-way stimuli are at least as effective in producing the type of "inhibition" referred to as alternating stimuli.

Another possible explanation of the phenomenon was a local modification of the nerve set up at, or between, the electrodes by the low-frequency stimulus. This local experimental modification of the condition of the nerve might affect it in its response to the high-frequency stimulus between the same electrodes. To test this possibility, in a certain number of experiments two pairs of electrodes were utilized as described above, *A* and *B* or *B* and *C*. First of all it was verified that the two pairs of electrodes were identical for the establishment of a steady state of heat production with an excitation of low frequency. In one experiment (May 18), during a steady state of activity set up by an alternating stimulus at the upper electrodes *A* and *B* (frequencies 100/sec.), the nerve was excited at the lower electrodes *B* and *C* at frequencies of 611/sec. and 912/sec. In both cases an abrupt negative deflection was produced followed by a positive deflection and a return to the steady state when the low-frequency stimulus at the upper electrodes *A* and *B* was substituted for the high-frequency stimulus at *B* and *C*. In the same experiment a stimulus of 912/sec. at the upper electrodes *A* and *B*, substituted for 100/sec. at the same electrodes, produced the same phenomenon, the negative deflection being more rapid than when *B* and *C* were employed. Some local effect, therefore, may occur, but it is not the cause of the phenomenon.



In the experiment of May 22, during a steady state with a stimulus of 48/sec. at the upper electrodes *A* and *B*, successive stimuli were applied as follows, all at 605/sec.: (1) at *A* and *B*, (2) at *B* and *C*, (3) at *A* and *B*. In all three cases, owing to the rather lower frequency, a very short positive deflection was observed followed by a large negative deflection. The same results were obtained in the experiments of May 24 and May 26. The phenomenon, therefore, cannot be explained by a local modification of the nerve at or between the electrodes of the conditioning stimulus. The distance from *B* to *C* was 12–15 mm., so it is very unlikely that shocks of low frequency at *B* could directly affect the nerve at *C* sufficiently to block it to subsequent high-frequency stimulation. The effect found in high-frequency stimulation at *C* must have been conditioned by low-frequency impulses previously reaching *C* from above, and not by the direct effect of the shocks.

If, during a steady state of heat production, due to prolonged excitation at low frequency, more rapid stimuli of various frequencies are applied, a graduated effect is obtained in the modification of the heat production. In one experiment (May 8), during a steady state produced by a stimulus of 112/sec., a stimulus of 705/sec. replacing it, caused a positive deflection followed by a large negative deflection. A stimulus of 1008/sec. produced a slight positive deflection followed by a negative deflection more rapid than the preceding. A stimulus of 1570/sec. produced a negative deflection immediate and abrupt. In another experiment (May 15) analogous results were obtained at frequencies of 305/sec., 605/sec., 813/sec. The same was found in eight experiments in which, not only the frequency was varied, but also the electrodes and the mode (one-way or alternating) of excitation. All the effects intermediate between a production of heat and its almost complete suppression can be obtained when a prolonged excitation at low frequency is changed to an excitation of high frequency. In every case the return to the excitation of low frequency causes a re-establishment of the previous state.

The duration of the preceding excitation of low frequency also has an influence on the thermal response of the nerve to a stimulus of high frequency. In one experiment (May 22) after 30 min. stimulation at 48/sec., the steady state of heat production not having been quite reached, an excitation of 605/sec. was applied. A small positive deflection was observed which lasted for 3 min. and was succeeded by a negative deflection. A return to the 48/sec. then caused a restoration of the previous state of steady heat production. After a total of 80 min. of low-frequency excitation the stimulus of 605/sec. was again applied, this time with an

immediate fall in the production of heat. In another experiment (May 23) a stimulus of 906/sec. maintained for 2 min., after 12 min. of stimulation

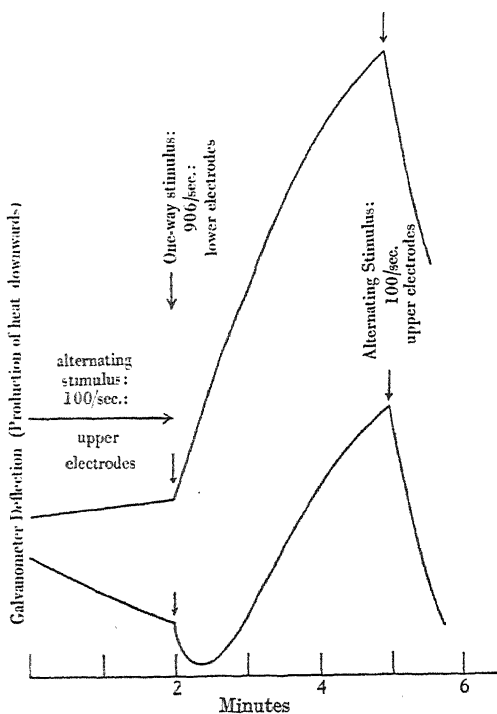


Fig. 3. Influence of the duration of the conditioning low-frequency stimulus (100/sec.) on the heat production of nerve when the frequency is suddenly raised to 906/sec. Experiment of May 24, 1933. The lower curve shows the application of the high-frequency stimulus to a nerve previously stimulated for 12 min. only at 100/sec., the steady state of heat production corresponding to this low frequency not yet having been attained. An immediate extra production of heat occurs, followed after 30 sec. by a diminution. The heat production rises again on returning to the lower frequency. The upper curve corresponds to the high frequency applied to the same nerve previously stimulated for 31 min. at the lower frequency, the steady state of heat production corresponding to the low frequency having already been reached. The diminution in heat production is immediate but the curve rises again when the frequency is again lowered. The low and the high-frequency stimuli were applied at two different pairs of electrodes, as described in the text, the conditioning low-frequency shocks being at electrodes more distant from the region of the nerve in which the heat production was measured. Heat production downwards as galvanometer deflection without analysis.

at 103/sec. which had not yet led to the establishment of a steady state, produced an immediate negative deflection of 60 mm. After a total of 30 min. of stimulation at 103/sec., a steady state of heat production being

now established, the same high-frequency excitation now gave an immediate negative deflection of 82 mm. Analogous results were obtained in the experiments of May 24 and 26 (Fig. 3). The production of heat by the

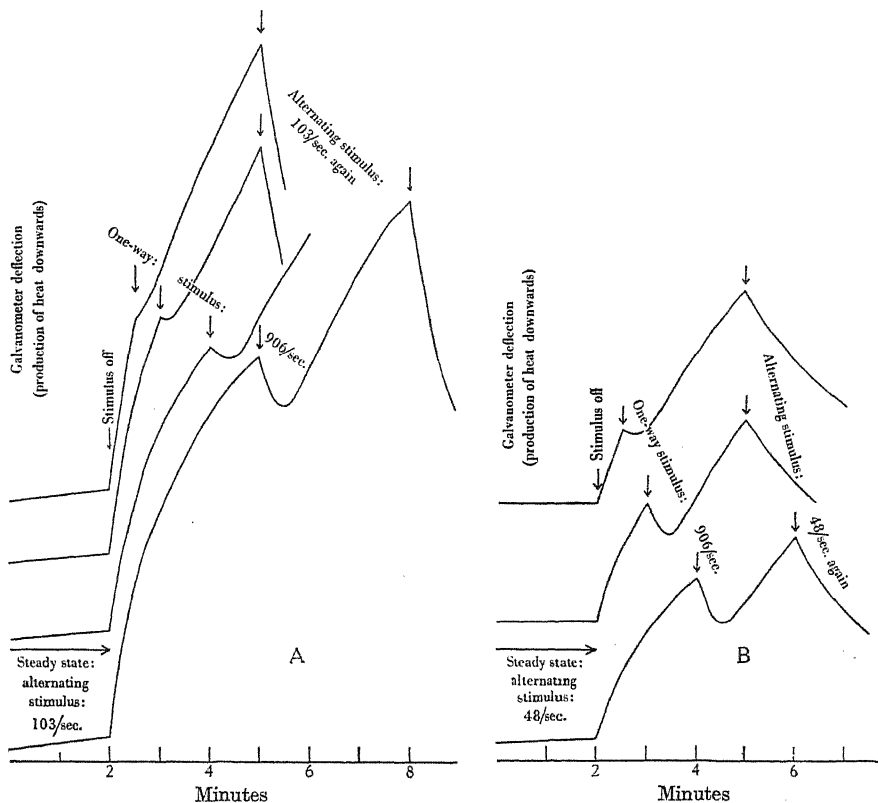


Fig. 4. Effect of an excitation of high frequency (906/sec.) on the heat production of nerve rested for various intervals after a previous prolonged stimulation leading to a steady state at low frequency. A, conditioning frequency 103/sec.; B, conditioning frequency 48/sec. Intervals of rest: A,  $\frac{1}{2}$ , 1, 2 and 3 min.; B,  $\frac{1}{2}$ , 1 and 2 min. Note that in B the response to the high-frequency stimulus is greater after a conditioning stimulus of low frequency than after one of a rather higher frequency. Heat production downwards as galvanometer deflection without analysis. Experiment of May 23, 1933. For discussion see text.

nerve, therefore, is more rapidly and intensely diminished as the result of an excitation of high frequency, when the duration of the low-frequency excitation which it replaces has been greater.

The frequency of the initial slow stimulus plays a similar part in the

response to a high-frequency stimulus following it. In the experiment of May 23 an excitation of 906/sec., lasting for 2 min., followed first an excitation of 100/sec. lasting for 12 min., then an excitation of 48/sec. lasting for 12 min. In the first case the negative deflection was 60 mm., in the second case it was 40 mm. Similar results were obtained in the experiment of May 22. For equal durations of prolonged excitation at low frequency the negative response to the same excitation of high frequency replacing it is greater the greater the frequency of the slow preceding stimulus.

In a last series of experiments the response was studied to an excitation of high frequency at various intervals after the suppression of a stimulus of low frequency. For example, in the experiment of May 23, after a stimulus of 103/sec. which had lasted for 45 min. and led to a steady state of heat production, the stimulus was suppressed altogether for 30 sec. A very rapid negative deflection was observed. A stimulus of 906/sec. was then applied and the negative deflection continued but less rapidly. The low-frequency stimulus was reapplied and the steady state was re-established. Excitation was suppressed for a minute with an immediate negative deflection. The high-frequency stimulus of 906/sec. was then applied again for 2 min.: the negative deflection stopped and then continued at a diminished speed. Later, following a steady state at low frequency, the nerve was rested for 2 min. and then an excitation of 906/sec. produced at first a small positive deflection and then a negative one. A rest of 3 min. caused an accentuation of the same phenomenon (Fig. 4). It seems, therefore, that the modification produced in the nerve, in respect of which it fails more or less to respond to a stimulus of high frequency, lasts for a certain time after the suppression of the prolonged stimulus of low frequency which caused it. The complete return of the power of the nerve to respond to a high-frequency stimulus probably does not occur before the nerve has completely recovered from its preceding steady excitation.

#### DISCUSSION.

The experiments reported show that prolonged excitation of a nerve by shocks of low frequency renders it incapable, to a greater or less degree, of responding to a stimulus of high frequency. This is not a question, however, in any ordinary sense of "fatigue." The nerve, which is no longer excitable by, or gives only a small response to, a stimulus of high frequency, responds to a stimulus of low frequency by a production of heat which is constant and may be continued for a very long time, or almost indefinitely. It seems rather that the nerve during the course of prolonged excitation at low frequency is put into a state in which it is

"inhibited" or partly inhibited, by a stimulus of high frequency. The state in which inhibition, instead of excitation, occurs when a rapid stimulus is applied, is reached only gradually, after a time of stimulation at low frequency of the order of 10, 20 or 30 min. The duration required depends upon the frequency of the slow excitation employed. The inhibitory effect is shown in all degrees as one increases progressively the frequency of the conditioning stimulus, its duration, and the frequency of the rapid stimulus tested.

Experiments of Hill [1932] and of Feng and Hill [1933] have shown that there is no simple relation between the frequency of excitation and the heat produced by the impulses set up. Another variable is involved, namely, the duration of the stimulus employed, and it now seems that the state of the nerve as affected by previous stimulation also must be taken into account. The heat per impulse is a function, not only of the frequency of excitation, but of the duration of previous stimulation and of the previous history and the state of recovery of the nerve.

One naturally asks whether the effect observed may not be due to a modification in the duration of the refractory period. Forbes and his collaborators [1923], Gasser and Erlanger [1925], and numerous other authors, have shown that if impulses follow one another in a nerve, at intervals short enough to make it necessary for them to travel in fibres incompletely recovered, the velocity of propagation and the size of the action current are modified: the refractory period is increased, the speed of propagation reduced, the size of the electric changes diminished. Such experiments have been concerned with the propagation of two impulses only, or of a series of impulses far shorter than we have considered in the present experiments.

The lengthening, however, of the refractory period, after a long interval of excitation at low frequency, is not sufficient alone to explain the "inhibition" to stimuli of high frequency. While admitting that a greater proportion of the shocks in the high-frequency stimulus would fall, in consequence of the lengthening of the absolute refractory period, within an interval of inexcitability of the nerve, it is difficult to understand why the response to an excitation of high frequency should not be (as it is in a nerve at rest) at least as great as to the excitation of low frequency. It seems, as Hill [1934] remarks, and as Verworn [1914] supposed for a series of shocks leading to Wedensky inhibition, that a stimulus *B* falling in the absolute refractory period of an impulse *A* must extend the refractory period, *i.e.* must render the nerve inexcitable by a following stimulus *C*, although *C* following *A* alone would have produced a response.

Fröhlich also (referred to by Lucas [1911]) supposed that "recovery" may be hindered by stimuli falling within the absolute refractory period: though Lucas himself could find no trace of such a relation with three stimuli applied to a previously resting nerve. The state in which the relation seems actually to occur is acquired by a nerve during slow steady stimulation at a rate to which it can respond indefinitely, and which does not therefore in any ordinary sense fatigue it. In a resting nerve, with a stimulus of short duration, the higher the frequency the greater the response.

This "inhibition" recalls in certain respects the phenomenon described under the name of Wedensky inhibition. When a muscle-nerve preparation has been modified by fatigue, or by a narcotic, stimuli of high frequency to the nerve no longer produce muscular response. The explanation generally given of this phenomenon involves the modification of the state of the neuro-muscular junction. Wedensky [see Lucas, 1911, p. 84] showed, moreover, that similar inhibition may occur in nerve alone, with local impairment of conduction. Tsai [1931] also has shown that a narcotized portion of nerve will allow impulses derived from a muscular sense organ to pass at a limited frequency but will arrest impulses of a higher frequency. In the present case the conditioning cause can scarcely be a local block since previous slow excitation at a point 12-15 mm. above the point tested renders the nerve liable to "inhibition" on high-frequency stimulation. H. and P. Davis [1932] have shown that the tension exerted by a muscle is a maximum for stimuli of low frequency and is diminished when the frequency is raised. These phenomena are probably all of the same nature.

It seems then, that when a nerve is subjected to prolonged steady non-fatiguing stimulation of moderate frequency, a condition is gradually set up in which a stimulus of high frequency produces a state of inhibition. During, and for some time after, this state of steady and constant excitation (which is presumably at least as representative of the normal physiological state as one of complete rest) the process of "recovery" from the refractory condition following a shock is different from that observed in the case of an isolated impulse, or during stimulation of short duration of whatever frequency applied to an otherwise resting fibre.

#### SUMMARY.

When a nerve is subjected to prolonged stimulation of low frequency (up to 100 shocks/sec.) it finally reaches a steady state in which the rate of heat production is constant. In this state, although in no ordinary sense fatigued, it is largely incapable of responding to an excitation of high

frequency of the order of 1000 shocks/sec. It remains, however, capable of responding by the same steady heat production on returning to the stimulus of low frequency. If, during a state of rest, the nerve is subjected to the same high-frequency excitation, it now gives an immediate maximal response. The phenomenon seems to be of the same nature as Wedensky inhibition, but it occurs in the nerve trunk.

The effect is not due to any local modification of the nerve at the exciting electrodes. It appears at a considerable distance from the point at which the conditioning stimulus of low frequency is applied, as is shown by the fact that the same failure to respond to a high frequency may occur at a distant electrode. The effect is the same whether the stimuli alternate in direction or are all "one-way."

All degrees can be observed between complete inhibition and a steady production of heat when the frequency of the secondary stimulus (the rapid one) is varied.

The fall in the response on the application of the higher frequency is greater and more rapid the higher the frequency of the basic conditioning stimulus and the longer the time of its application. When the low-frequency conditioning stimulus is stopped and an interval of recovery allowed, the response to the high-frequency stimulus is greater the greater the interval of intervening rest.

It is difficult to give an explanation of the phenomenon on the basis of what is known about the passage of a single impulse, or of a few isolated impulses, in an otherwise resting nerve. As the result of prolonged steady activity, not so intense, however, as to lead to fatigue, the processes of "recovery" from the refractory state following a shock are modified, so that a continuously refractory condition may be set up by shocks succeeding one another at sufficiently short intervals.

My warmest thanks are due to Prof. A. V. Hill for suggesting the subject of this work and for providing the material and the counsel necessary for its realization. My thanks are due also to Mr J. L. Parkinson for his help and advice.

#### APPENDIX.

By A. V. HILL.

The state of "inhibition" described in the preceding paper is not propagated away from the stimulating electrodes, as is shown by the following experiment. A thermopile was prepared with four electrodes, *A*, *B*, *C* and *D*, the distances being as follows:  $AB=2.2$  mm.,  $BC=16$  mm.,

$CD = 3.4$  mm.,  $D$  to nearer end of thermopile = 18 mm. A high frequency stimulus (1000/sec.) initially producing a maximal response was applied at  $A$  and  $B$  until the response was largely reduced; without stopping the stimulus at  $A$  and  $B$  a low frequency stimulus (100/sec.) was then applied at  $C$  and  $D$  and a good response was obtained.

Another experiment was made as follows. A short (16 sec.) high-frequency stimulus at  $A$  and  $B$  initially caused a much greater response than a similar low-frequency stimulus at  $C$  and  $D$ . When the latter had been continued for some time, the former applied for 16 sec. during the continuance of the latter always gave a slight positive effect, showing (a) that there was not complete failure at  $A$  and  $B$ , and (b) that nothing like "inhibition" was being propagated from  $AB$  to  $CD$ . If, however, the low frequency at  $CD$  was turned off while the high frequency at  $AB$  was turned on, there was a negative response, showing that the high frequency was now producing a smaller effect than the low frequency, therefore a much smaller effect than it did initially.

It seems, therefore, that the "inhibition" referred to by Bugnard is connected with the process of excitation, each element of the high-frequency stimulus causing, or maintaining, a higher threshold or a prolonged refractory period. The condition in which this "inhibition" may be observed on high-frequency stimulation can be produced by a steady stream of impulses propagated from a distant point. The "inhibition" itself, however, is local. One cannot "inhibit" low-frequency impulses starting at  $CD$  by high-frequency stimulation at  $AB$ ; though one can block (at  $CD$ ) low-frequency impulses starting from  $AB$  by high-frequency stimulation at  $CD$ .

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## THE ANAEROBIC BREAKDOWN OF CARBOHYDRATE IN THE ISOLATED VENTRICLE OF THE FROG.

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It has been shown by Clark, Gaddie and Stewart [1931] that the isolated heart of the frog beating in air or oxygen derives at least part of its energy from non-carbohydrate sources. There is definite evidence of the usage of nitrogenous material, but not of the usage of fat, even though the R.Q. may be very low. Under anaerobic conditions, however, the frog's heart resembles striped muscle in depending ultimately upon carbohydrate as the source of energy though the breakdown of phospho-creatine is probably the primary source [Clark, Eggleton and Eggleton, 1932]. Clark, Gaddie and Stewart [1932] have also shown that the continued power of the heart to contract in the absence of oxygen depends upon two factors: the presence of carbohydrate which may be broken down to lactic acid, and the supply of a sufficiently alkaline perfusion fluid to neutralize and remove the lactic acid formed. Thus a neutral perfusion fluid prevents the contraction from continuing for longer than a few minutes, since the lactic acid quickly accumulates in the heart. On the other hand, a fluid buffered to pH 8 with  $\text{NaHCO}_3$  or  $\text{Na}_2\text{HPO}_4$  allows the heart to contract, even without added glucose, for some hours at the expense of its own carbohydrate stores; for in this case the lactic acid is neutralized and removed by the perfusion fluid.

When the heart, perfused by alkaline Ringer fluid, has been exhausted anaerobically, it can be completely revived by the addition of glucose, an observation made by Freund and König [1927] and confirmed by Clark, Gaddie and Stewart [1932]. It is evident that the same restorative effect should be exerted by any substance which can replace glucose

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as an anaerobic source of energy, and by any substance which is an intermediate in the breakdown of carbohydrate to lactic acid, provided that its conversion to lactic acid liberates a sufficient amount of energy to allow of contraction. The frog's heart, exhausted by continued anaerobic contraction with an alkaline perfusion fluid, is thus an exceedingly convenient preparation for the study of carbohydrate metabolism in muscle. It is also, of course, suitable for investigating the action of various muscle poisons, of which iodoacetic acid is, at present, the most important.

Like skeletal muscle, cardiac muscle is prevented from contracting anaerobically by iodoacetic acid, which, however, does not abolish aerobic contraction [Clark, Eggleton and Eggleton, 1932].

The possibilities for cardiac muscle may therefore be summarized as follows:

A. Unexhausted heart contracting in oxygen, and oxidizing its own stores of carbohydrate and protein.

B. Exhausted heart contracting in oxygen, depleted of its stores of carbohydrate and available protein, benefited by addition of glucose and amino acids.

C. Unexhausted heart contracting in nitrogen, converting carbohydrate to lactic acid which must be removed from the cells by an alkaline perfusion fluid.

D. Exhausted heart contracting in nitrogen, depleted of carbohydrate, benefited only by sugar or intermediates in carbohydrate breakdown to lactic acid.

E. Unexhausted heart contracting in oxygen and poisoned by iodoacetic acid, probably using only amino acids.

F. Unexhausted heart contracting in nitrogen, poisoned by iodoacetic acid, probably dependent solely on phosphagen breakdown.

G. Exhausted heart contracting in oxygen, poisoned by iodoacetic acid, depleted of available nitrogenous material, probably benefited by amino acids and by lactic acid but not by carbohydrate.

The experiments described in this paper are concerned with conditions D and F; conditions E and G are being studied at present, and will form the subject of a further communication.

#### METHODS.

*Rana esc.* (Hung.) was used. The perfusion of the isolated ventricle was carried out with a cannula in the auriculo-ventricular opening, and arranged so that isometric records could be taken when desired. The ventricle was stimulated electrically at any desired rate (see Appendix).

The apparatus was of similar type to that illustrated by Clark [1927, Fig. 1]. Air and nitrogen from a fine jet could be bubbled through the perfusion fluid, and the current of gas served to stir the fluid. The nitrogen was rendered oxygen-free by passing it over heated copper, and recontamination was guarded against by closing the cannula with a rubber stopper fitted with a tube for the escape of the gas.

The Ringer fluid used had the following percentage composition: NaCl 0.65, KCl 0.015,  $\text{CaCl}_2$  0.012,  $\text{NaHCO}_3$  0.05. When gas was bubbled through this fluid the pH rose to about 8.5. Glucose and other substances in solution were introduced into the perfusion fluid through a hole in the rubber stopper, closed by a glass rod when not being used.

The sugars and glutathione used in our experiments were B.D.H. reagents. Methyl glyoxal was made by the method of Hoffmann and

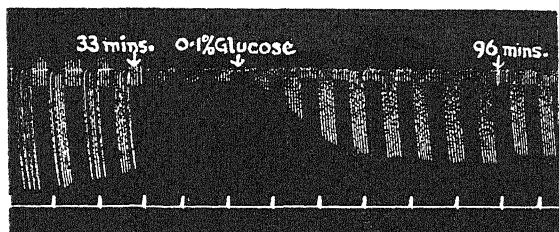


Fig. 1. Typical recovery of an exhausted ventricle on addition of glucose. (Anaerobic.)

Neuberg [1930], dihydroxyacetone and glyceric aldehyde by the method of Hersant and Linnell [1931]. Iodoacetic and bromoacetic acids were synthesized from chloroacetic acid and precipitated from hot benzene solution by addition of petrol ether. The remaining iodo compounds were synthesized by Miss D. Mowat who kindly supplied us with samples.

#### RESULTS AND DISCUSSION.

(a) *The effect of glucose.* Fig. 1 is a typical record of the gradual exhaustion of the ventricle perfused by alkaline Ringer in an atmosphere of nitrogen and of the recovery on addition of glucose. For about an hour the heart contracts in nitrogen as strongly as in oxygen, but thereafter the contractions gradually lose strength until they finally disappear. The time required for this total exhaustion varies from as little as 2 to as much as 6 hours, according to the amount of glycogen available, the rate of stimulation, and the frequency with which the perfusion fluid is changed.

The importance of the first two of these factors is obvious. With regard to the third, it seems that the perfusion fluid washes out a part of some substance necessary for proper contraction, so that changing the perfusion fluid two or three times definitely hastens the exhaustion. More frequent changing, however, has no further effect. The nature of this substance will be considered further in section (c).

The recovery on addition of glucose is rapid, but is more so when, as in Fig. 1, the ventricle has not been allowed to become completely exhausted. Evidently, if the heart has completely stopped the diffusion of the sugar through the narrow cannula is relatively slow. When recovery is complete, *i.e.* in about 10 min. after addition of glucose, changing the perfusion fluid leads to a rapid re-exhaustion of the ventricle which can then be recovered again by glucose. This process of alternate exhaustion and recovery can be repeated many times on the same ventricle. Advantage was taken of this in controlling the experiments described later in this paper, for a trial of any substance was always sandwiched between two successful glucose recoveries.

(b) *Other carbohydrates.* Although it is known that the intact animal is capable of utilizing a variety of carbohydrates, it is not certain whether some, or all, of the tissues are able to use them directly, or whether they must first be converted to glycogen in the liver. It would seem *a priori* that glucose, fructose and mannose might all be used directly by muscle, since they possess a common enol form. However, Maclean and Smedley [1912] found that the hearts of the dog and rabbit, in presence of oxygen, utilized glucose and mannose fairly rapidly, and galactose to a slight extent, but maltose, fructose and xylose, not at all. The negative result with fructose was also obtained by Steinberg [1927] using rabbit's heart muscle, and by Ashford [1933] with brain. Working with embryonic tissues under anaerobic conditions, Dickens and Greville [1932] found that some were able to convert only glucose to lactic acid, while others were able to convert fructose as well.

In the isolated frog's ventricle the results with fructose were completely negative, whereas mannose restored the power of contraction as rapidly and completely as did glucose itself (Fig. 2). The other carbohydrates tested all gave negative results—*pentoses*: arabinose, ribose, xylose; *hexoses*: galactose; *disaccharides*: maltose, lactose, sucrose; *polysaccharides*: glycogen, starch.

Although Freund and König reported recovery after addition of glycogen to the exhausted heart, we have never succeeded in obtaining any trace of recovery. The size of the glycogen molecule makes its diffusion

into the cell somewhat unlikely, and of course such a diffusion is an essential preliminary to recovery. We have found, however, that minute traces of glucose are capable of bringing about some degree of recovery, and it is just possible that Freund and König used a specimen of glycogen slightly contaminated with glucose.

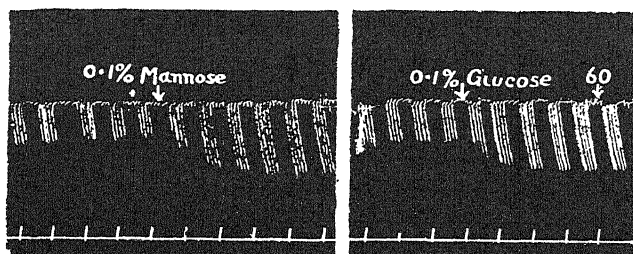


Fig. 2. The action of mannose on an exhausted ventricle (in absence of oxygen) compared with the action of glucose on the same ventricle.

(c) *Non-carbohydrate substances.* Since amino acids are capable of acting as sources of energy under aerobic conditions [Clark, Gaddie and Stewart, 1931], and in view of the statement by Freund and König that glycine and alanine restored the activity of the exhausted anaerobic heart, these substances were tested in the isolated ventricle exhausted in nitrogen. The results, however, were completely negative. Glycine and alanine were also found to be without effect on the strength of the

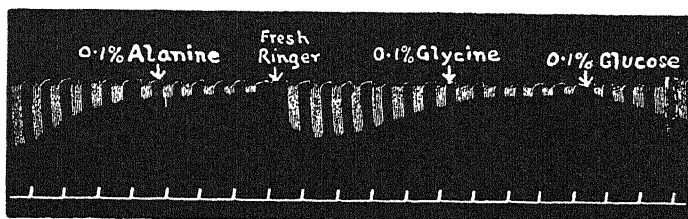


Fig. 3. Failure of alanine and glycine to cause recovery of the exhausted ventricle in nitrogen. Glucose restores power of contraction.

contraction of the unexhausted heart whether working in air or nitrogen (Fig. 3).

Clark, Gaddie and Stewart [1931] found that the addition of soaps to the perfusion fluid caused a rise in oxygen consumption by the isolated frog's heart without any change in the R.Q. The effect of soaps on the

exhausted heart was somewhat similar. Fig. 4 shows that the addition of 0.1 p.c. sodium oleate to the exhausted heart produced a temporary recovery. It is to be noted, however, that this recovery with soaps could be made once only by each heart, and could not be repeated many times as in the case of recoveries with glucose. Evidently the fatty acid, unlike glucose, is not acting as a source of energy. This result agrees with Clark's [1913] hypothesis that soap restores some constituent of the cell which is removed by perfusion.

(d) *Possible intermediates in the breakdown of carbohydrate.* The conversion of carbohydrate to lactic acid most probably involves the splitting of the hexose molecule into two triose molecules. As a preliminary stage, it seems that the hexose is first esterified with phosphoric acid, and there

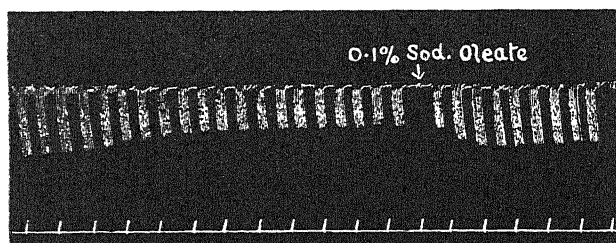


Fig. 4. Slight recovery on addition of sodium oleate to the fluid perfusing the ventricle partly exhausted in nitrogen.

is evidence that the triose molecules are also produced in the form of phosphoric esters. In this connection of course the substances to be considered are glyceric aldehyde and dihydroxyacetone.

Trial of these two trioses on the exhausted ventricle, however, gave very disappointing results. Dihydroxyacetone was without effect; glyceric aldehyde did appear to increase the force of contraction, but the response to stimulation became very irregular and ceased altogether within a few minutes. Larger concentrations of glyceric aldehyde were definitely toxic, at once preventing the ventricle from responding to stimulation. The heart recovered, however, if the glyceric aldehyde was washed out and replaced by glucose. Glucose gave no recovery unless the glyceric aldehyde was first removed (Fig. 5).

It may be objected that the true intermediates in carbohydrate breakdown are the phosphoric esters of these trioses and not the trioses themselves. It is arguable, however, that if the heart muscle is able to

use glucose directly, *i.e.* to esterify it, it should be able to do the same with the trioses. This is not necessarily the case, since the esterified state of the trioses may be due merely to their production from a hexose diphosphate. It must be admitted, in fact, that further work is necessary on this point. Our results so far as they go, however, suggest that while minute amounts of glyceric aldehyde may be utilized by the heart muscle, larger concentrations are definitely toxic.

There remains the possibility that the trioses prepared synthetically exist in inactive forms very different from these produced and utilized in the enzymatic breakdown of hexose. This point need not be stressed, for the possibilities of keto-enol changes in these compounds are well known, but it is obvious that in face of these objections our negative results cannot

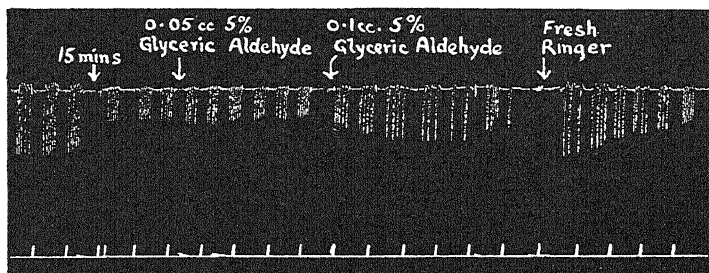


Fig. 5. The action of glyceric aldehyde on the exhausted ventricle in nitrogen, increase in the force of contraction, but irregularity in the response to stimulation.

be taken as eliminating these two compounds from consideration as intermediates in lactic acid formation.

Whatever triose or trioses may be produced from hexose, their further breakdown most probably furnishes either methyl glyoxal or pyruvic acid. The former could of course be produced from either dihydroxyacetone or glyceric aldehyde by intra-molecular change, the simultaneous oxidation of one carbon atom and reduction of another within the same molecule, the net result being the elimination of water. Pyruvic acid formation involves more than this, the addition of an extra atom of oxygen. Consequently, since the reaction, if it takes place at all, does so anaerobically, a second molecule of some kind must be reduced. In other words, there is the probability of the simultaneous formation of glycerol (or glycerophosphoric acid).

Methyl glyoxal and sodium pyruvate were tested on the exhausted ventricle; the former produced a rapid and sustained recovery, the latter

was without effect (Fig. 6). It may be noted incidentally that the recovery in presence of methyl glyoxal was less complete than that produced by glucose, and that, after a partial recovery was produced by the addition of methyl glyoxal, a further recovery was produced by glucose.

This result lends support to the view that methyl glyoxal is an intermediate in the formation of lactic acid from carbohydrate. It must be borne in mind, however, that glyoxalase may conceivably not form a part of the enzyme system concerned in carbohydrate utilization. In their essentials, the experimental results merely indicate that the energy liberated in the conversion of methyl glyoxal to lactic acid is adequate for contraction and that the muscle can use it for that purpose. We know that aerobically the muscle is capable of utilizing energy from a variety of sources. It may well be that the same is true under anaerobic conditions, but that ordinarily the breakdown of sugar is the only one available, and that the supply of methyl glyoxal allows an utterly abnormal mechanism to function. We may say therefore that the possibility of the utilization of methyl glyoxal is proved but that the inclusion of methyl glyoxal as an intermediate in carbohydrate breakdown remains unproved. Further consideration of this matter is best deferred until a later section of the paper.

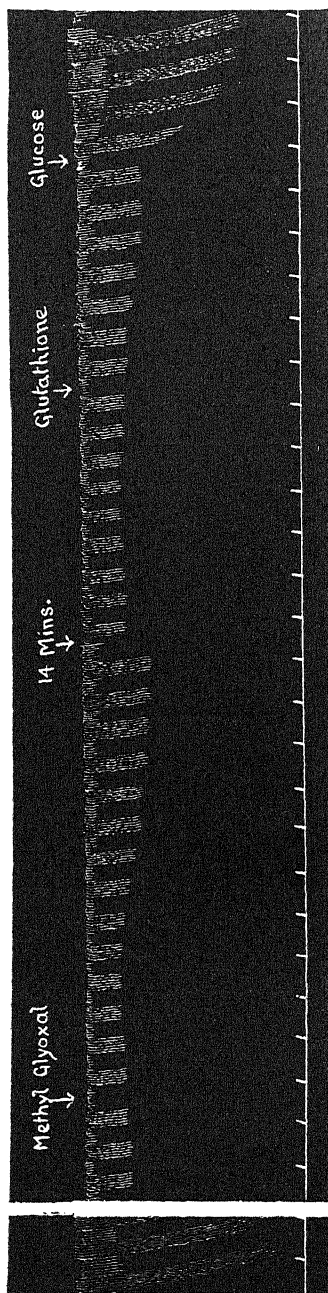


Fig. 6. The effect of methyl glyoxal on the exhausted ventricle in nitrogen, partial recovery, increased somewhat by glutathione, and converted to full recovery by glucose.



The conversion of methyl glyoxal to lactic acid involves the addition of a molecule of water, the reduction of the keto group and the oxidation of the aldehyde group. Thus no other substance (except water) is involved. Pyruvic acid on the other hand is reduced to lactic acid so that under anaerobic conditions some other molecule must be oxidized. In the formation of pyruvic acid glycerol is probably produced, and since there is no evidence of its accumulation in the muscle, it must be supposed to be the hydrogen donator in the reduction of pyruvic acid. Consequently the fact that pyruvic acid alone is unable to restore the activity of the ex-

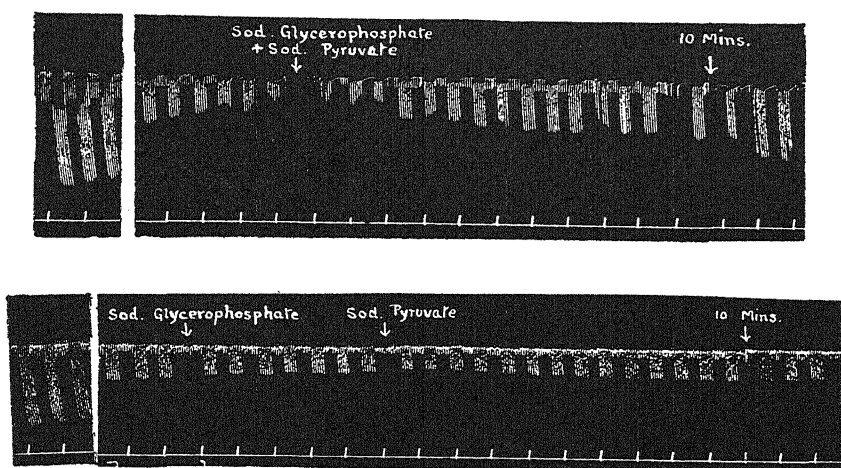


Fig. 7. Partial recovery of the ventricle exhausted in nitrogen by means of equimolecular mixture of sodium pyruvate and sodium glycerophosphate. The upper tracing shows the most complete recovery obtained, the lower shows the usual response. Note that sodium glycerophosphate alone is without effect. Sodium pyruvate alone (not shown) is equally ineffective.

hausted ventricle must be regarded as inconclusive in eliminating pyruvic acid as an intermediate in lactic acid formation. We therefore tested an equimolecular mixture of sodium pyruvate and sodium glycerophosphate. As Fig. 7 shows a partial recovery was produced, the effect never being as great as that of methyl glyoxal and sometimes little more than bare maintenance of weak contractions. Here then we have definite evidence in favour of the chain of reactions which involve the production of pyruvic acid and glycerol—the chain postulated by Embden and by Meyerhof for the breakdown of carbohydrate in skeletal muscle extracts and in yeast fermentation.

In order to gain further evidence as to the power of the heart muscle to form phosphoric esters other than those of hexose, we further tested equimolecular mixtures of sodium pyruvate and glycerol, which gave no restoration of the power to contract.

A number of other substances which have been suggested as possible intermediates in lactic acid formation were tested, but with uniformly negative results: succinic acid, hydroxybutyric acid, aceto-acetic acid, fumaric acid, maleic acid, acetic acid, acetaldehyde, ethyl alcohol and ethylene glycol.

It is especially noteworthy in the experiments with glycerophosphoric acid and pyruvic acid (as with methyl glyoxal) that after recovery had proceeded to its maximum, a still further increase in the strength of contraction was produced by the addition of glucose. If it be accepted that glycerophosphoric acid and pyruvic acid together form one stage in lactic acid production it is justifiable to conclude further that with the utterly abnormal amounts present the enzymes concerned in the reaction must have been completely saturated. Moreover, their presence in such concentration would probably suffice to prevent their further production from added sugar. It follows then that if glucose is to produce an additional effect, it must do so by being broken down in some other way than that involving the production of these two substances. The alternative though (in our opinion) less probable explanation is that the energy liberation in the reaction: glycerophosphoric acid + pyruvic acid = lactic acid + triose phosphate, accounts for a fairly small part of the total energy of the change glucose  $\rightarrow$  lactic acid; and that glucose in spite of the high concentration of glycerophosphoric and pyruvic acids is still converted to these two substances with liberation of more available energy.

A similar argument, of course, applies to the experiments with methyl glyoxal, so that both series suggest the existence of two routes for the conversion of carbohydrate to lactic acid.

(e) *The action of iodoacetic acid and related compounds.* Under aerobic conditions low concentrations of sodium iodoacetate (*i.e.* conc. of about 1 : 20,000 in the perfusing fluid) have little or no effect for several hours, but when oxygen is excluded, the heart ceases to contract after about 7 min. (Fig. 8). Re-admission of oxygen to the paralysed heart (together with massage if contraction has entirely ceased) causes a complete revival, and this process of alternate stoppage by iodoacetic acid in absence of oxygen, and revival by oxygen can be repeated many times. The concentration of sodium iodoacetate necessary to stop the heart in absence of oxygen is about 0.005 p.c. or 0.25 millimol. Lower concentrations than

this are either without action or diminish the power of anaerobic contraction gradually without completely abolishing it (except perhaps after so long that the stoppage may be due as much to lack of carbohydrate as to the poisonous effect of the iodoacetic acid). Concentrations higher than 0.005 p.c. do not increase the rapidity of the action, but high concentrations are rapidly toxic even in the presence of oxygen.

Sodium monobromoacetate acts as rapidly as sodium iodoacetate and in equal concentrations; sodium monochloroacetate, however, is without effect, even in concentrations of 2.5 millimol. This finding is of importance in view of (a) the observation of Dudley [1931] that iodoacetic acid inhibits glyoxalase, (b) the results of Lohmann [1932] indicating that

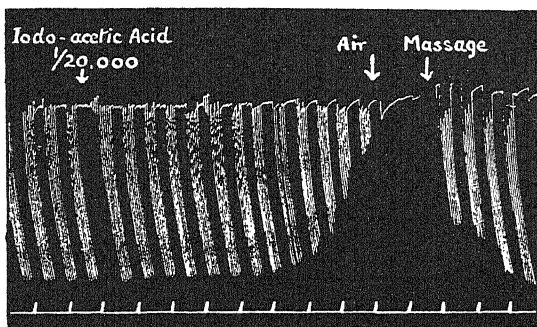


Fig. 8. The action of iodoacetic acid on the fresh ventricle under anaerobic conditions. In this case the heart was allowed to stop completely before oxygen was admitted, and it was necessary to re-start contraction mechanically.

iodoacetic acid inactivated the co-enzyme of glyoxalase and of Jowett and Quastel [1933] indicating that this co-enzyme is glutathione; (c) the finding by Dickens [1933 a] that *in vitro* iodoacetic acid reacts with glutathione, liberating hydriodic acid.

Since it has been found [Dickens, 1933 b] that glutathione reacts, in slightly alkaline solution, with all three mono-halogen derivatives of acetic acid, liberating the halide acid, the results on the frog's ventricle suggest that iodo- and bromoacetic acids have some further action in addition to their inactivation of co-glyoxalase. Monochloroacetic acid reacts with glutathione at only about 1/10 the velocity with which monobromoacetic acid reacts. Nevertheless, it is able, in blood, to cause an appreciable slowing of the rate of disappearance of glucose, in a concentration about three times as great as the effective concentration of bromo- and iodoacetic acids [Mowat and Stewart, 1934].

Mono-iodo propionic acids, both  $\alpha$  and  $\beta$  isomers, are without action on the frog's ventricle even in high concentrations. Iodoethyl alcohol,  $\text{CH}_2\text{I} \cdot \text{CH}_2\text{OH}$ , is also unable to prevent contraction under anaerobic conditions. These facts are peculiar since all three substances have been found to have an appreciable effect in inhibiting the conversion of glucose to lactic acid in blood [Mowat and Stewart, 1934].

(f) *The effect of glutathione on the ventricle poisoned by iodoacetic acid.* Since methyl glyoxal was found to restore activity to the ventricle exhausted in absence of oxygen, and since strong evidence had been produced by Lohmann, Jowett and Quastel, Dickens, etc., that iodoacetic acid reacted with and destroyed glutathione, thereby preventing the conversion of methyl glyoxal to lactic acid, we attempted to

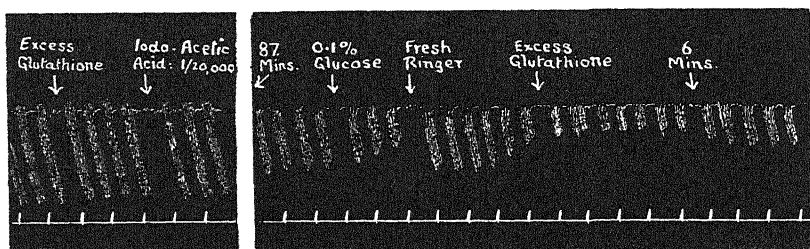


Fig. 9. Ventricle in nitrogen. Addition of iodoacetic acid to fluid already containing glutathione reduced the contraction only very slowly, so slowly that the effect was due largely to exhaustion of carbohydrate stores. Addition of glucose at this stage further reduced the force of contraction. Removal of glucose by washing and addition of more glutathione brought about slow recovery.

restore activity to the ventricle paralysed by iodoacetic acid, by addition of an excess of reduced glutathione to the perfusion fluid.

In our first experiment glutathione (neutralized) was added to the perfusion fluid of a heart contracting anaerobically to make a 1.25 millimol. solution. There was no effect on the strength of contraction. Sodium iodoacetate was then added in the usual concentration (0.25 millimolar) and at the end of 15 min. the heart was still contracting with almost undiminished strength. A second addition of iodoacetate equal to the first was still insufficient to cause paralysis (Fig. 9).

This experiment, however, was really unsatisfactory, since the  $p\text{H}$  of the fluid was that at which glutathione and iodoacetic acid rapidly interact *in vitro*, so that the glutathione in the perfusion fluid was probably acting merely by preventing the iodoacetate from ever reaching the muscle cells. A second experiment was therefore made. Sodium iodo-

acetate was added in the usual amount, and, when the ventricle had almost ceased to contract, the perfusion fluid was removed and replaced by fresh Ringer's solution containing glutathione. The ventricle at once began to contract, and the strength of contraction gradually increased to about half the normal, at which it remained for over an hour, when the experiment was stopped. The observation was of course repeated several times, and it was shown that mere changing of the perfusion fluid without supplying glutathione was quite unable to restore activity to the ventricle. In fact repeated change of the perfusion fluid, the heart being meanwhile kept contracting in oxygen, was quite incapable of removing the effect of iodoacetic acid, and exclusion of oxygen at any time resulted in the rapid cessation of contraction (Fig. 10).

In this second type of experiment the glutathione is evidently reversing the effect of the iodoacetic acid by some action within the muscle itself. This action may and probably does consist in restoring the muscle glutathione previously destroyed by the iodoacetic acid. The recovery after addition of glutathione is never complete, however, and this fact, taken in conjunction with others recorded in the preceding pages, suggests that iodoacetic acid has other effects than that of inactivating co-glyoxalase and that these other effects are irreversible by any means yet discovered.

Cysteine, it may be noted, is capable of preventing the action of iodoacetic acid subsequently added to the perfusion fluid, but is quite unable to revive the ventricle already poisoned by iodoacetic acid.

It is remarkable that if glucose be added to the perfusion fluid of a heart poisoned by iodoacetic acid and revived by glutathione, the heart rapidly ceases to contract. (The simultaneous presence of glutathione and glucose has no effect on the unpoisoned heart.) The poisonous effect of glucose under these conditions is removed by merely changing the perfusion fluid, *i.e.* removing the glucose (Fig. 10).

As an explanation of this phenomenon it may be suggested that one (at least) of the intermediates in the conversion of glucose to lactic acid is toxic except in minute concentrations (*cf.* the experiments with glyceric aldehyde), and that iodoacetic acid affects the mechanism of lactic acid formation from this intermediate. Recovery by glutathione may be supposed sufficient to allow the removal of the toxic substance as quickly as it is formed from the normal sugar reserves of the heart. Addition of what is in effect an enormous amount of readily available sugar then causes the accumulation of the toxic substance at a rate much greater than that at which it can be removed. It may be recalled here, however,

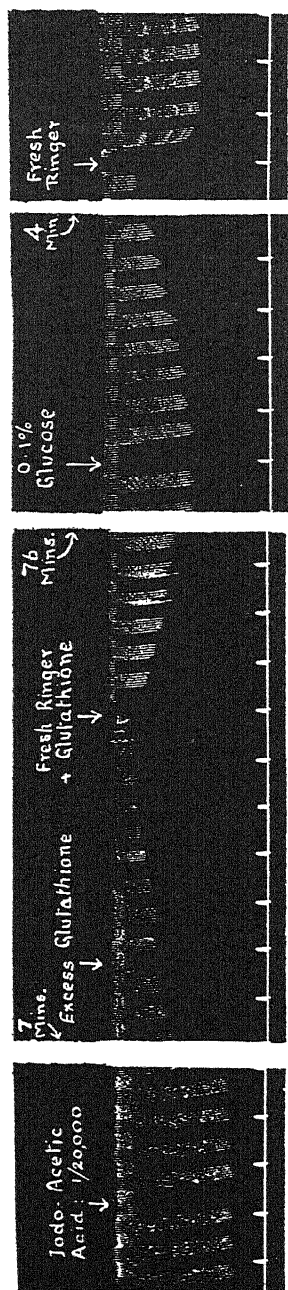


Fig. 10. Ventricle in nitrogen, poisoned by iodoacetic acid, restored by addition of glutathione and change of perfusion fluid, and again poisoned by addition of glucose.

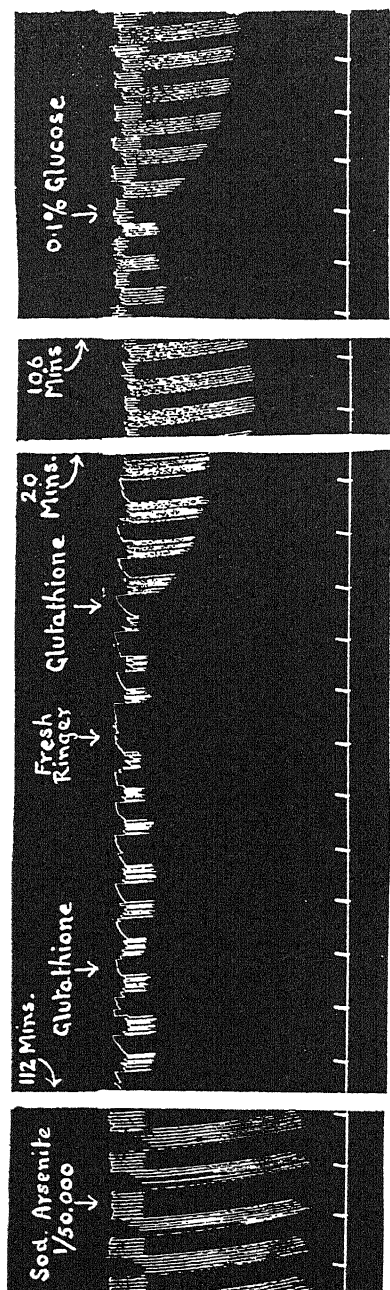


Fig. 11. Ventricle in nitrogen, slowly poisoned by sodium arsenite, restored by glutathione and change of perfusion fluid, and, after slow exhaustion (of carbohydrate stores), restored by glucose.

that glucose was incapable of restoring activity to the exhausted heart in the presence of glyceric aldehyde.

(g) *The action of arsenic.* Voegtlin and his collaborators [1923, 1931] have adduced evidence in favour of their hypothesis that trivalent arsenic combines with reduced glutathione and that the latter has therefore a protective action against arsenic in, *e.g.*, rats, rabbits, trypanosomes, etc.

If the two substances do combine, it follows that small quantities of arsenic should exert an effect on muscle similar to that of iodoacetic acid. It should, in fact, prevent the anaerobic contraction of muscle if the contraction depends upon the activity of glyoxalase, provided it is not added in amounts too great to be dealt with by the glutathione present. Excessive amounts of arsenic, of course, would in all probability act as a general enzyme poison. Under the conditions we are imagining:

(1) the muscle should be capable of contracting when supplied with oxygen but not in absence of oxygen (showing that the enzymes concerned in non-carbohydrate oxidation or aerobic carbohydrate breakdown are intact);

(2) the power of anaerobic contraction should be restored by reduced glutathione.

We find (Fig. 11) that sodium arsenite, at a concentration of 1/50,000 in the perfusion fluid, gradually abolishes the power of contraction in the isolated ventricle stimulated under anaerobic contraction. The action is much slower than that of iodoacetic acid, but is much more rapid than that of mere exhaustion of the carbohydrate reserves. Admission of oxygen restores the power of contraction to the ventricle poisoned in this way, and the power is again lost if the oxygen is excluded.

When anaerobic conditions are maintained, the poisoned ventricle is not revived by washing with normal Ringer nor by the addition of glucose. (In much higher concentrations arsenic prevents the heart from contracting even in oxygen—*i.e.* it acts as a general enzyme poison. Under these conditions, its effect on aerobic contraction can be removed by repeated washing.) It is, however, revived by glutathione (best after change of perfusion fluid to remove excess of arsenic) and the power of contraction is then retained until the carbohydrate stores are exhausted in the normal way, when glucose brings about a further recovery (Fig. 11).

The heart poisoned by this low concentration of arsenic and restored by glutathione appears to be quite normal. Changing the perfusion fluid several times, so as to remove circulating glutathione, does not decrease

the strength of contraction, so that the glutathione supplied must have penetrated the tissue and become fixed in the same way as the normal glutathione of the muscle.

It is evident that under the experimental conditions the main action of arsenic consists in its interaction with glutathione. It is not a general enzyme poison, and since glucose produces its normal effect after recovery of the poisoned heart by glutathione and exhaustion of carbohydrate stores, there is no sign of an effect on any other factor concerned in glucose breakdown, *i.e.* no accumulation of toxic intermediates. Since glutathione is known to be the co-enzyme of glyoxalase, it follows that methyl glyoxal is an intermediate in the formation of lactic acid from glucose in cardiac muscle.

It seems probable indeed that in cardiac muscle this is the main route available for the anaerobic breakdown of glucose, since the production of lactic acid *via* triose phosphates and pyruvic acid is stated [Meyerhof, 1933] to be independent of glutathione. It is possible that the pyruvic acid route is primarily that taken by glycogen, since in the experiments recorded here the glycogen reserve was already depleted at the time recovery with glutathione took place, as evidenced by (a) the partial nature of the recovery, and (b) the relatively short time before addition of glucose became necessary.

Moreover, since the production of methyl glyoxal is probably not interfered with, it follows that either a very low concentration of methyl glyoxal stops further breakdown of carbohydrate, or the energy liberated in the conversion of glucose to methyl glyoxal is very small.

(h) *Discussion of results.* It seems highly probable that there are at least two mechanisms for the breakdown of carbohydrate to lactic acid in living tissues. On the one hand there is a considerable body of evidence in favour of the supposition that methyl glyoxal is formed as an intermediate stage, and on the other hand much experimental work indicates that pyruvic acid occupies a similar position.

Methyl glyoxal is of course rapidly converted to lactic acid by a great many tissues since the enzyme concerned—glyoxalase—is very common. This, however, is not to be regarded as strong evidence in favour of the production of methyl glyoxal from sugar. Much more important in this respect are the demonstration that methyl glyoxal can be utilized in place of sugar by the exhausted heart, the recovery in presence of glutathione of the heart poisoned by iodoacetic acid or by arsenic, and the isolation of methyl glyoxal from muscle pulp under various conditions. Thus Case and Cook [1931] detected small amounts of the substance in muscle pulp



under both aerobic and anaerobic conditions; Toeniessen and Fischer [1926], Arayama [1928], Sym [1931], Inoue [1931], Barrenschéen *et al.* [1931, 1933], and others have all demonstrated the power of muscle pulp to produce methyl glyoxal (often in good yield) from hexose diphosphate. In the presence of muscle pulp, pancreatic extract, and iodoacetic acid, Barrenschéen, Braun and Dreguss found that glycogen also gave rise to methyl glyoxal.

Pyruvic acid has also been detected in muscle pulp under the same conditions as methyl glyoxal [*e.g.* Case and Cook, 1931]; Meyerhof [1933] has shown that muscle contains enzymes capable of producing lactic acid from a mixture of pyruvic acid and glycerophosphoric acid, and that pyruvic acid is produced during the breakdown of sugar in muscle extract; and we have found that these two substances are together capable of restoring some power of contraction to the frog's ventricle exhausted anaerobically.

It seems to be established therefore that both of these substances are formed during the production of lactic acid from carbohydrate. It follows either that the one must be converted into the other or that there are two ways in which lactic acid may be formed from carbohydrate.

The chain of reactions, methyl glyoxal  $\rightarrow$  pyruvic acid  $\rightarrow$  lactic acid, is conceivable, although Jowett and Quastel [1933] have adduced evidence to support the supposition of direct addition of water to an addition compound of methyl glyoxal and glutathione. Case considers that methyl glyoxal is converted to pyruvic acid and that this is the source of pyruvic acid in muscle glycolysis. His evidence for this, however, is of a purely negative character, in that he has failed to detect any other source of the observed pyruvic acid. Nevertheless, he failed to obtain pyruvic acid from added methyl glyoxal and was driven to postulate an active form of the latter in spite of the fact that synthetic methyl glyoxal readily gives rise to lactic acid.

It is worth mentioning, too, that Neuberg and Kobel [1930] described the production of pyruvic acid from hexose diphosphate by yeast as occurring later than that of methyl glyoxal. It is obvious, therefore, that the possibility of methyl glyoxal and pyruvic acid lying on the same line of breakdown must be considered, though it can be definitely accepted only after more positive evidence has accumulated. On this view, however, it becomes difficult to explain Meyerhof's statement that dialysed muscle, in presence of added adenosinetriphosphoric acid and magnesium, produces lactic acid from carbohydrate—*i.e.* under conditions in which glyoxalase cannot act.

The second possibility—that there are two separate enzyme systems each converting carbohydrate to lactic acid in its own way—is supported by much evidence and explains many phenomena which would otherwise be obscure. It is to be noted that the two systems would not necessarily act on the same carbohydrate and that they might well occur in varying proportions in different tissues.

Moreover, they might assume different relative degrees of importance in the same tissue at different times or under different conditions.

Recently Meyerhof [1933] has advanced strong evidence in favour of the view that carbohydrate is converted *via* hexose diphosphate to triose phosphates, thence to pyruvic and glycerophosphoric acids, and so to lactic acid. In so doing he refuses to admit the possibility of methyl glyoxal having any share in the reaction, basing his objection chiefly on the following facts:

(a) Glutathione is essential for the conversion of methyl glyoxal to lactic acid.

(b) Lactic acid is produced from glucose even by dialysed muscle extracts, after the addition of adenosine—triphosphoric acid and magnesium.

He ignores, however, the considerable body of positive evidence in favour of methyl glyoxal, and completely overlooks the possibility of a double mechanism. Yet the view suggested in the present paper is directly supported, *inter alia*, by the work of Ashford on brain tissue [1933]. Ashford found that glycogen, hexose diphosphate, hexose monophosphate, glucose and mannose are all converted to lactic acid by brain tissue, as also are methyl glyoxal (more rapidly than glucose) and pyruvic acid (less rapidly than methyl glyoxal). If, however, any one of the group: glycogen, hexose diphosphate and hexose monophosphate, was incubated with brain tissue along with glucose or mannose, the lactic acid produced was the sum of the amounts obtained from the two substances separately (when lactic acid formation was proceeding at the maximal rate). Ashford draws the obvious deduction that there must exist two enzyme systems, one dealing with glucose or mannose, the other with glycogen or hexose phosphate.

The evidence from our own work in support of this view may be summarized as follows:

(1) Recovery of the exhausted heart by methyl glyoxal or by pyruvic acid (+ glycerophosphoric acid) is incomplete.

(2) After maximal recovery by these substances, glucose brings about a further recovery.

The recovery of the ventricle on addition of the substances suspected of being lactic acid precursors is evidence that they can be utilized. The fact that the recovery is only partial might be considered to indicate that the heart was in an abnormal or damaged condition, were it not for the further observation that after their removal, glucose invariably brought about a complete recovery. The quantities of the intermediates added to the perfusion fluid were obviously greatly in excess of any amounts which might be formed normally during the breakdown of carbohydrate, and it is reasonable to suppose that they were sufficient to saturate the enzymes concerned. Consequently the additional recovery given by glucose added in presence of intermediates is strong evidence of the existence of alternative routes.

It might be objected that the extra recovery due to glucose is attributable to energy liberated by the breakdown of glucose to methylglyoxal or pyruvic acid as the case may be. Unfortunately, the published values for the heat of combustion of methylglyoxal differ too widely for calculation of the energy released during the earlier stages to be of any value. On the whole, the indications are that this energy must be very small. In the case of pyruvic acid, the position is complicated by the fact that some other compound must be simultaneously oxidized, and again, one can do no more than speculate on the magnitude of the energy changes involved.

(3) Monochloroacetic acid,  $\alpha$ - and  $\beta$ -iodopropionic acids, and iodoethyl alcohol are without action on the isolated frog's ventricle contracting anaerobically. They are, however, capable of inhibiting glycolysis in blood [Mowat and Stewart, 1934]. This observation of course indicates a difference between the relative importance of the two hypothetical systems in cardiac muscle and in blood.

(4) Even in large excess, glutathione is unable to restore full power of contraction to the ventricle poisoned by iodoacetic acid. Yet it completely abolishes the power of iodoacetic acid to inhibit glycolysis in blood [Mowat and Stewart, 1934].

(5) Glutathione when added to the fluid perfusing a frog's ventricle which had been poisoned in the absence of oxygen with the minimum amount of trivalent arsenic, completely restored the power of anaerobic contraction. It is important to notice, however, that the amount of arsenic added was too small to produce a general enzyme poisoning as was shown by the power of the poisoned ventricle to contract normally in oxygen. In other words the arsenic, like iodoacetic acid, had specifically inhibited the enzymes responsible for the breakdown of carbohydrate to lactic acid.

Even the two-route hypothesis is insufficient to explain the fact that the ventricle which has been poisoned and recovered by glutathione behaves differently towards glucose according to whether the poison used was iodoacetic acid or trivalent arsenic. The toxic action of glucose upon a ventricle poisoned by iodoacetic acid (and recovered by glutathione) can be explained by postulating the formation of a toxic intermediate. No such toxic intermediate is formed, however, in the case of arsenic. Since, however, arsenic stops all lactic acid formation (the ventricle, so poisoned, is not recovered by glucose alone), it follows that arsenic has a double action. It inhibits glyoxalase by combining with glutathione just as does iodoacetic acid; it differs from iodoacetic acid, however, in inhibiting the primary stages of carbohydrate breakdown at an earlier point. The toxic intermediate, on the basis of Meyerhof's explanation of the action of iodoacetic acid and our own findings with respect to dihydroxyacetone and glyceric aldehyde, may possibly be phosphoglyceric acid, which we have not yet had the opportunity of testing on the frog's ventricle.

It is not suggested that the evidence presented in this paper is sufficient to prove the existence of a double line for the breakdown of carbohydrate to lactic acid. It is, however, suggested that this hypothesis is to some extent supported by evidence and that it offers the best explanation, at the moment, of a number of puzzling phenomena.

#### SUMMARY.

1. The isolated ventricle of the frog, exhausted of carbohydrate by continued contraction in the absence of oxygen, is revived by the addition to the perfusion fluid of glucose or mannose, but not fructose, galactose, arabinose, ribose, xylose, maltose, lactose, sucrose, glycogen, starch, glycine, or alanine.

2. Under similar circumstances, sodium oleate and sodium linoleate produced temporary recovery which, unlike that caused by glucose and mannose, could not be repeated after re-exhaustion of the same ventricle.

3. Under similar circumstances, methyl glyoxal produced a partial recovery, convertible to full recovery by the addition of glucose; pyruvic acid, sodium glycerophosphate, and dihydroxyacetone were separately without effect; pyruvic acid and sodium glycerophosphate together produced slight recovery, increased by glucose to full recovery; and dl-glyceric aldehyde increased the force of contraction temporarily, but exerted a toxic effect.

4. The isolated ventricle, contracting in absence of oxygen, was rapidly stopped by iodo- and bromoacetic acids in concentrations of about 0.25 millimol.; monochloroacetic,  $\alpha$ - and  $\beta$ -iodopropionic acids and iodoethyl alcohol were without effect even in much higher concentrations. The effect of iodoacetic acid was not removed by frequent changing of the perfusion fluid.

5. The isolated ventricle, poisoned by iodoacetic acid in the absence of oxygen, was restored to activity by addition to the perfusion fluid of excess of reduced glutathione. The recovery was not complete, and the addition of glucose rapidly abolished contraction. Cysteine was able to prevent the action of iodoacetic acid but not to restore activity to a ventricle which had been poisoned by iodoacetic acid.

6. Sodium arsenite, in a concentration of 0.25 millimol., slowly abolished the power of contraction of the isolated ventricle in absence of oxygen but not in presence of oxygen. The ventricle, so poisoned, partially recovered the power of anaerobic contraction on the addition of reduced glutathione, and the further addition of glucose completely restored the power of contraction.

7. The bearing of these results on the mode of carbohydrate breakdown is discussed, and it is suggested that there may be two routes for the formation of lactic acid—one *via* methyl glyoxal, and the other *via* pyruvic acid.

We wish to acknowledge gratefully the interest which Prof. A. J. Clark has taken in the progress of this work. We have also to thank the Moray Research Fund of this University for a grant in aid of the expenses.

#### APPENDIX.

A modification of the magnetic tipper previously described [Condon, 1913] was devised by Mr N. E. Condon to give electrical stimuli at regular intervals. The outer jacket of a Liebig condenser was arranged to give a constant flow of water (Fig. 12, *L*) into a bucket, *A*, so making and breaking at regular intervals an electric contact, *E*. The platinum contacts of the older apparatus were replaced by a piece of platinum wire fixed to the bucket arm and arranged to dip into a cup of mercury at *E*. This provides the make-and-break of the primary circuit, and the mercury switch has the advantage over the platinum contact of allowing a longer time for the passage of current through the primary coil.

The essential modification of the older apparatus consisted in a small vulcanite platform *V*, on which were mounted two metal cups *F* contain-

ing mercury, and a hinged rod *G* provided at one end with a means *H* of shorting the two cups and, at the other end, a small metal plate *P*. Through a  $\frac{3}{16}$  in. hole in this plate passed a curved piece of steel banjo wire of gauge 29 (K), with a slight kink near the end. The curvature of the wire was so arranged that when the bucket was empty, the wire just

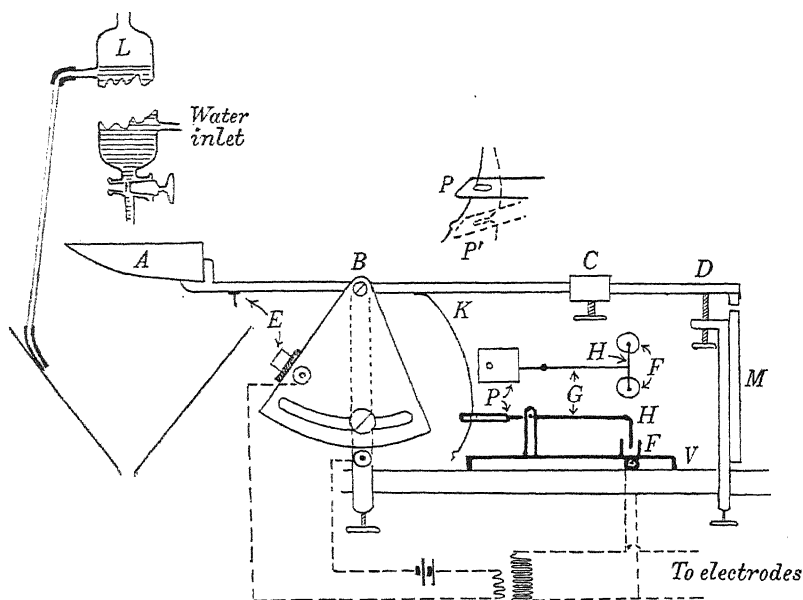


Fig. 12. Modification of Condon's magnetic tipper, designed to give electric stimuli at regular intervals.

touched the end of the hole remote from the hinge. When, however, the bucket was full and tipping, the wire engaged the opposite side of the hole. Thus for a short period during the return journey of the bucket, the wire depressed the metal plate and so lifted the wire which shorted the metal cups (which formed a switch in the secondary circuit).

The wiring is sufficiently shown in the diagram.

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## GLYCOGEN STORAGE AND LÆVULOSE TOLERANCE.

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CARBOHYDRATE tolerance tests, especially when done with lævulose, are thought to give some indication of liver function [Isaac, 1920; Isaac and Siegel, 1928; von Bergmann, 1932; Stroebe, 1932; Kimball, 1932, etc.]. Hofmeister [1890] was the first to point out that dogs who had been starved for a long time developed glycosuria after the ingestion of carbohydrates (hunger-diabetes). Barrenschéen [1914] showed by transfusion methods that less glycogen than normal was formed in a liver if it were from a starved animal and contained little glycogen. Bang [1913] demonstrated a high and prolonged blood-sugar curve following glucose ingestion in rabbits after a period of starvation. Staub [1922] found a similar type of blood-sugar curve after fasting in man. In view of the previous work of Barrenschéen, this type of blood-sugar curve was taken to mean that the liver cell could not form glycogen when it contained little itself [Lesser, 1920; Staub, 1922; Lichtwitz, 1930]. Isaac [1920] showed that after ingestion of lævulose the blood sugar in normal man rises only slightly, whereas with a damaged liver there is a considerable rise.

The small normal rise of blood sugar in man after ingestion of lævulose was believed to be due to unchanged lævulose [Isaac, 1920]. The micro-method of estimating lævulose introduced by van Crefeld [1927] showed, however, that it is always mainly due to glucose [van Crefeld and Ladenius, 1928; and others]. In the case of experimental damage to the liver (chloroform, hydrazine sulphate) and disease in man the larger rise is partly due to unchanged lævulose but much more to glucose, in some cases indeed to it alone; Corley, 1929; Heinecke and Peters, 1930; Steinitz, 1932; and others]. The unchanged lævulose when found is in all probability due to the failure of such a liver to convert lævulose fully into glucose, a conversion which was shown by Mann and Magath [1921] to be a function of the normal liver. The increase in



glucose was assumed, on the basis of Barrenscheen's experiments, to be due to the inability of a liver containing too little glycogen to make any more. Vogt [1932], under Stroebe, held the definite view that "only a liver rich in glycogen can deal adequately with large quantities of carbohydrate. A raised and prolonged blood-sugar curve after lævulose justifies the conclusion that the liver contains little glycogen." Buettner and Neuhaus [1931], Stroebe [1932], Steinitz [1932] and others consider this type of blood-sugar curve as evidence of the inability of the damaged liver to form or store glycogen. There is no experimental evidence to justify these opinions.

The following paper, therefore, deals with the problem whether a lack of glycogen in the liver cells impairs the power of forming glycogen after ingestion of lævulose and whether the blood-sugar curve is determined by the readiness with which glycogen can be formed or stored in the liver.

#### METHODS.

Groups of male rats were submitted to different periods of starvation. The blood sugar and the average resting glycogen content of the liver and muscles were determined, and then blood sugar, liver and muscle glycogen estimated at intervals after the administration of a standard dose of lævulose.

Each series consisted of nine or ten animals of about 150 g. body weight which were kept under the same conditions two weeks before the experiment. Some (3 to 5) were then killed, while the rest received the sugar and were killed one at the end of each hour up to the sixth hour after it. The standard meal consisted of about 500 mg. of lævulose which was dissolved in 1 c.c. of water and introduced into the stomach by a fully filled 1 c.c. syringe fixed to the end of a stomach tube. At the end of the experiment the syringe was again fully filled with the lævulose solution employed in the experiment, then emptied into a 200 c.c. flask, made up with water and the exact amount of lævulose estimated in an aliquot part by Hagedorn-Jensen's method. Immediately after the death of the rat the liver and the skinned left leg were frozen in liquid air, and the glycogen content estimated by Pflüger's method. The muscular tissue remained almost perfectly intact by removing the leg from the pelvis. Blood was obtained from the heart with filter paper or by bleeding into a dish containing oxalate crystals. Estimation of blood sugar was made by Hagedorn-Jensen's method. The amount of lævulose absorbed was calculated by subtraction of the sugar still found in the alimentary tract after death from the total amount administered.

In order to determine the l  vulose content of the gut, the whole alimentary tract was removed between ligatures and cut in pieces. The fluid was expressed from the segments which were then washed and all the fluid so obtained was filtered through glass wool. The sugar content of an aliquot part of the filtrate was then determined. No appreciable sugar content was found by this method in the empty alimentary tract.

## RESULTS.

### *L  vulose absorption from the gut.*

The amount of sugar absorbed has been stated by Cori [1925, 1926] to depend very much on the length of the previous fasting period. He found that 77 mg. of l  vulose were absorbed per hour per 100 g. rat with a previous fasting period of 48 hours, whereas with only 24 hours more than 100 mg. were absorbed. In the present experiments (Table I) no marked difference in absorption was found in rats starving for 12, 24, 48 or 96 hours. It was, however, considerably faster in winter, average 200 mg. in the first hour, than it was in the summer, when it averaged 131 mg. The average for all rats examined was found to be 174 mg. in the first hour, 142 mg. in the second and 125 mg. in the third. Absorption was complete at the end of the third hour. The values of blood sugar were not affected by the rate of absorption.

TABLE I. L  vulose absorption from the alimentary tract.

	Rate of absorption (in mg. per 100 g. rat)		
	1st hour	2nd hour	3rd hour
Rats starving 12 hours	180	148	128
" " 24 "	156	130	—
" " 48 "	149	135	115
" " 96 "	164	142	123
Rats killed during winter	202	157	127
" " " summer	131	119	114
Average of all rats	174	142	125

### *Glycogen formation in the wall of the intestine.*

If glycogen were formed in the intestinal wall during the absorption, the subsequent breakdown of this glycogen might contribute to the raising of the blood sugar. Accordingly estimations of glycogen in the intestinal wall were made; the amounts found were negligible and showed little or no change during and after l  vulose absorption.

*The blood sugar.*

In this and the following sections the results are best set out by grouping the experiments thus.

Group I consisted of well-fed rats without a long previous fasting period. They were fed with 1 g. lævulose per 150 g. body weight on the eve of the experiment (12 hours before it) and then kept starving. In this group 52 rats were employed, 25 of them being killed at different intervals after the lævulose meal. In these latter the average increase of blood sugar was 7 mg. per 100 c.c.; in 68 p.c. of them not more than 10, in 32 p.c. between 10 and 50 mg. per 100 c.c.

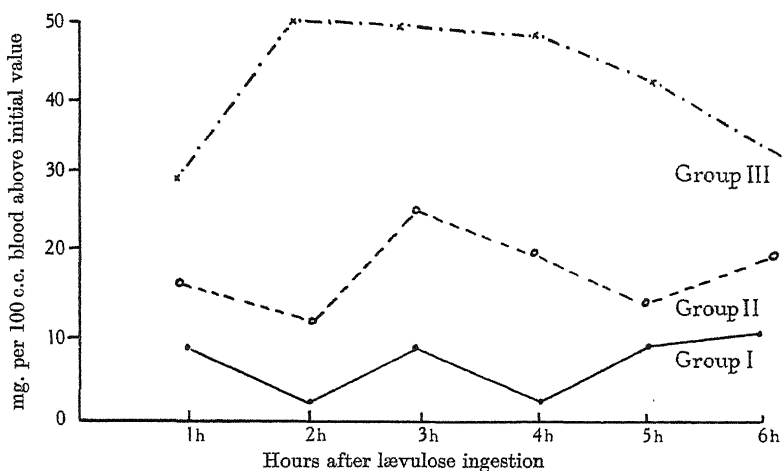


Fig. 1. Blood-sugar increase after lævulose meal. Figures given in mg. per 100 c.c. above initial level. Group I: well-fed rats. Group II: short fasting period. Group III: long fasting period.

Group II, 70 rats starving 12–48 hours, of which 47 were examined after getting lævulose. In these the average rise of blood sugar was 18 mg. per 100 c.c.; in 19.2 p.c. less than 10, in 78.7 p.c. between 10 and 50, and in 2.1 p.c. more than 50 mg. per 100 c.c.

Group III, 46 rats fasting for 96 hours, of which 28 were examined after lævulose. In these the average rise of blood sugar was 41 mg. per 100 c.c., in 3.6 p.c. of them less than 10, in 71.4 p.c. between 10 and 50, and in 25 p.c. above 50 mg. per 100 c.c.

The blood-sugar curves of groups I and II (Fig. 1) show a moderate increase during the course of the experiment, whereas the curve of group III corresponds to a "high and prolonged blood-sugar curve"

[Vogt, 1932]. If the statements referred to above were correct groups I and II ought to have shown a good initial glycogen content in the liver and good formation and storage after l  vulose, whereas a lack of liver glycogen in the resting rats and a deficiency of glycogen formation and storage should have been found in the third group.

*The glycogen formation in the liver.*

*Group I.* This consisted, as already mentioned, of rats which were fed on the eve of the experiment with l  vulose and then kept starving. This procedure was adopted because when animals were killed without any starvation period or after a fasting period of four hours the glycogen in the liver varied very widely. By the method adopted livers were obtained which showed a good resting glycogen content. There was a considerable difference in the glycogen content of different series which may well be accounted for by difference in the season and in external circumstances. In any one series, however, the resting liver glycogen content was found to be of approximately the same value (Table II).

TABLE II. Resting glycogen content in the livers of rats.  
Figures given in g. per 100 g. liver.

Group I, well-fed rats. Group II, short fasting period. Group III, long fasting period.

	Group I	Group II	Group III
Series S	4.90	0.14	0.26
	4.40	0.12	0.18
	5.79	0.25	0.36
Series J	2.41	0.32	0.16
	2.68	0.42	0.39
	2.60	0.07	0.52
	2.19	0.07	0.34
Series W	6.16	0.08	0.54
	5.86	0.06	0.54
	7.03	0.07	0.88
		0.15	0.08
Series V	3.19	0.04	0.15
	3.64	0.08	0.21
	3.60	0.06	0.53
		0.13	0.39
		0.14	0.12
		0.12	1.18
		0.08	0.13
		0.15	
		0.23	
		0.10	
		0.15	
		0.19	
		0.12	
		0.16	
Average		0.14	0.39

After the standard dose of lævulose the average content of glycogen found in the liver (Table III) decreased in the course of the experiment. The most marked decrease was observed in series with the higher initial glycogen values, above 5 p.c. With an initial glycogen value below that figure there was a slight increase in the second and third hour, but a marked decrease occurred in the later hours of the experiment. As a control experiment water only was given. This was followed by a rapid and progressive decrease in liver glycogen. After the end of absorption (fourth hour of experiment) the glycogen in the liver was in every series actually less than at the beginning of the experiment.

TABLE III. Glycogen formation in the liver after 0.5 g. lævulose.

Figures given in g. per 100 g. liver above resting value.

+ indicates increase, - indicates decrease.

Group I, well-fed rats. Group II, short fasting period. Group III, long fasting period.

Time	Group I		Group II		Group III	
	Glycogen	No. of rats	Glycogen	No. of rats	Glycogen	No. of rats
1st hour	-0.64	3	+0.80	7	+0.43	5
2nd hour	-0.64	4	+2.05	9	+2.29	5
3rd hour	-0.40	3	+2.27	7	+3.05	5
4th hour	-1.57	3	+2.57	6	+3.74	4
5th hour	-1.71	3	+2.04	5	+4.01	5
6th hour	—	—	+1.89	5	+2.97	4

*Group II.* The liver glycogen after a short fasting period was found to be practically constant, usually below 0.2 and never exceeding 0.5 p.c. After the dose of lævulose the average amount of glycogen deposited in the liver was found to be 0.80 after 1 hour and 2.05 p.c. after 2 hours. The glycogen formation was generally smaller than the average in those rats in which absorption from the gut was low. After 4 hours 25 p.c. of the sugar absorbed was recovered as glycogen in the liver. The average absolute glycogen content after that period was 2.57 p.c. which is only slightly less than the absolute average figure of the first group (2.84) in spite of the considerable difference in the resting glycogen of these two groups.

*Group III.* The glycogen values after prolonged fasting were found to be practically constant and definitely higher than those in group II (Table II). A strong positive acetone reaction was present in the urine at the beginning of the experiment.

After the dose of lævulose the rate of glycogen formation in the liver during the first hour was poor, the average found being 0.46 p.c. in contrast with 0.80 in group II. At the end of the second hour it was the same as in group II (Table III); at the end of the fourth hour it was

3.74 p.c., which corresponds to 33 p.c. of the sugar amount absorbed, both these figures being higher than those of groups I and II.

The results given so far show that the amount of glycogen already in the liver does not determine the capacity of the liver to build up glycogen, as was suggested by Barrenschæen's experiments. The rats of group II, although they had but little glycogen in the liver, showed good glycogen formation, whereas the rats of group III with a somewhat higher resting glycogen value formed definitely less in the first hour. The store of glycogen built up after the end of the absorption period (fourth hour) was in both groups II and III better than in group I with a high initial glycogen value. Furthermore, the results do not confirm the explanation of the blood-sugar curve after lævulose hitherto given. According to the blood-sugar curves, groups I and II should have had a good store of glycogen in the liver to start with, and after lævulose have increased it considerably, whereas the reverse should have held for group III. It is, however, evident that group II had the smallest amount of glycogen in the liver before the dose of lævulose and group I the smallest amount after it; the best glycogen formation was found in rats of group III. When the increase of blood sugar is compared with the glycogen deposition in the liver (Table IV) it is seen that a high rise of blood sugar does not correspond to a small glycogen formation in the liver nor a small one with a large. The blood-sugar curve after lævulose has in these experiments no relation to the liver glycogen.

TABLE IV. Increase in liver glycogen in relation to the blood-sugar rise.  
Increase in liver glycogen in p.c. of lævulose absorbed.

Increase in blood sugar mg. per 100 c.c.	1st hour		2nd hour		3rd hour		4th hour		5th hour		6th hour	
	Gly- cogen	No. of rats	Gly- cogen	No. of rats	Gly- cogen	No. of rats	Gly- cogen	No. of rats	Gly- cogen	No. of rats	Gly- cogen	No. of rats
0-10	9	9	22	4	18	2	5	4	7	3	10	3
10-50	12	9	27	10	21	10	27	7	30	7	22	6
Above 50	13	1	31	2	42	1	27	2	24	1	—	—

*The glycogen formation in the muscles.*

*Group I.* The initial glycogen content of the muscles was approximately the same for all rats of this group of well-fed animals in spite of the considerable differences in the amount of liver glycogen in the different series (Table V). The average glycogen formation after lævulose was considerable; a comparatively large amount of glycogen was already deposited in the first hour after lævulose which was in all probability due to the breakdown of liver glycogen (Table VI).

*Group II.* The resting glycogen in the muscles of this group was low, as it was in the liver. After lævulose ingestion a good glycogen formation was found which increased every hour until the maximum was reached in the fourth hour of the experiment when absorption was finished. No appreciable difference is found in the average rate for the whole experiment between groups I and II (Tables V and VI).

TABLE V. Resting glycogen content in the muscles of rats.

Figure given in g. per 100 g. muscle weight.

Group I, well-fed rats. Group II, short fasting period. Group III, long fasting period.

Group I	Group II	Group III
0.31	0.16	0.20
0.31	0.17	0.24
0.36	0.14	0.24
0.32	0.14	0.36
0.38	0.15	0.38
0.31	0.20	0.19
0.22	0.18	0.20
0.40	0.15	0.32
0.26	0.28	0.21
0.31	0.22	0.27
0.27	0.15	0.26
0.41	0.19	0.12
0.26	0.22	0.23
0.26	0.18	
0.32	0.16	
0.24		
0.28		
Average	0.31	0.18
		0.25

TABLE VI. Glycogen formation in the muscle after lævulose.

Figures given in g. per 100 g. muscle above resting value.

+ indicates increase, - indicates decrease.

Group I, well-fed rats. Group II, short fasting period. Group III, long fasting period.

Time	Group I		Group II		Group III	
	Glycogen	No. of rats	Glycogen	No. of rats	Glycogen	No. of rats
1st hour	+0.12	3	+0.06	6	-0.01	5
2nd hour	+0.06	5	+0.08	8	+0.06	5
3rd hour	+0.13	5	+0.11	4	+0.09	5
4th hour	+0.13	5	+0.18	6	+0.07	4
5th hour	+0.13	3	+0.08	3	+0.05	4
6th hour	+0.12	2	+0.12	5	-0.02	4
Average	+0.12	23	+0.10	32	+0.04	27

*Group III.* The average initial glycogen content in this group held, as in the case of the liver, an intermediate position between the figures for groups I and II. No glycogen formation occurred during the first hour; the maximum was found after 3 hours and was about half as much as the maximum in group II. At each hour the figures are lower than

those for either group I or II, the average for the whole experiment being only one-third (Tables V and VI).

It must be noted that the increase of muscle glycogen is small like the initial glycogen content. The variations observed in the resting glycogen content in the liver, especially if this is low, do not affect the results after l  vulose because comparatively large quantities of glycogen are newly formed. The same variations, when they occur as they do, in the resting muscle glycogen are much more liable to affect the result owing to the comparatively small amount of glycogen formed. The number of animals, however, from which these averages are calculated is such that the figures certainly indicate a diminished power of building up glycogen in the muscles of rats that have fasted long (group III).

There is a definite relationship between the formation of muscle glycogen and the corresponding increases in blood sugar (Table VII). Rats showing a marked increase in blood sugar (above 50 mg. per 100 c.c.) show little if any formation of glycogen in the muscle; the amount formed is largest when the blood sugar rises least. The capacity of the muscle to build up glycogen seems, therefore, to have a definite relation to the blood-sugar curve. The blood sugar after l  vulose is high when the muscles cannot form glycogen.

TABLE VII. Increase in muscle glycogen in relation to the blood-sugar rise after 0.5 g. l  vulose meal.

Figures given in g. per 100 g. muscle weight above resting value and in mg. per 100 c.c. blood. Where the - sign appears there is no increase but a diminution.

Increase in blood sugar	1st hour		2nd hour		3rd hour		4th hour		5th hour		6th hour	
	Gly- cogen	No. of rats	Gly- cogen	No. of rats	Gly- cogen	No. of rats	Gly- cogen	No. of rats	Gly- cogen	No. of rats	Gly- cogen	No. of rats
0-10	0.12	5	0.10	5	0.12	4	0.15	5	0.17	2	0.16	3
10-50	0.03	9	0.06	8	0.11	10	0.11	6	0.09	6	0.03	7
Above 50	-0.08	1	0.01	3	0.02	1	0.03	2	0.05	2	—	—

### *The effect of thyroxine.*

The following experiment points to a similar conclusion. Abderhalden and Wertheimer [1930] showed that after thyroxine the livers of rats are unable to form glycogen. A series of seven rats were, therefore, injected daily with 0.5 mg. thyroxine (Schering) for 7 days. The animals were then kept fasting 12 hours before giving the l  vulose. The liver glycogen of the fasting rats was very low, 0.06 p.c. Following l  vulose ingestion there was hardly any increase, the maximum after 3 hours being 0.16 p.c. The muscle, however, the initial glycogen content of



which was also very low (0.09 p.c.), had risen after 3 hours to a maximum, 0.25 p.c., an increase similar to that observed in rats of group II. There was no marked rise in the blood-sugar level. This result is very much against the old explanation that the failure of the liver to form glycogen accounts for the rise of blood sugar after lævulose, while on the contrary the relation between glycogen formation in the muscles and blood sugar is the same as that in the experiments recorded above. With larger doses of thyroxine the blood sugar tends to rise considerably after lævulose [Kugelman, 1930] and the glycogen formation in the muscles of rats becomes poor [Abderhalden and Wertheimer, 1930].

#### SUMMARY.

1. It has been commonly held that (*a*) the power of the liver to form glycogen is impaired when its store of glycogen is low; and that (*b*) an excessive rise of blood sugar after taking lævulose is due to the inability of the liver to form and store glycogen. The experimental basis of these opinions has been critically examined.

2. The power of the liver to form glycogen has been shown not to be impaired when its store of glycogen is low.

3. An excessive rise of blood sugar after lævulose has been found to be associated neither with a low glycogen store nor with inability of the liver to form and hold glycogen.

4. On the other hand such a rise of blood sugar after lævulose was associated with little or no glycogen formation in the muscles.

5. The results suggest that inability, not of the liver, but of the muscles to build up glycogen is at least one factor of importance in an excessive rise of blood sugar after lævulose. The clinical observation that such a blood-sugar curve is given in disease of the liver is not disputed, nor is it incompatible with the above results if the anabolism of glycogen in the muscles is influenced by the metabolic changes which carbohydrates undergo in the liver.

I wish to express my thanks to Prof. von Bergmann for many kindnesses shown to me while working at the Charité Krankenhaus, Berlin, and for facilitating this investigation in every way. I am also extremely indebted to Dr A. F. Hurst for his encouragement and advice which has enabled me to correlate my researches and present them in this form; and to Mr Spurrell and Dr S. L. Simpson for very helpful criticism.

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## THE CARBON DIOXIDE BALANCE BETWEEN THE MATERNAL AND FETAL BLOODS IN THE GOAT.

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### INTRODUCTION.

THE possibility of carbon dioxide secretion at or in the placenta was not entertained by the earlier investigators, although the same question with regard to oxygen was most seriously debated [Zuntz, 1877; Cohnstein and Zuntz, 1884; Charpentier and Butte, 1888; Butte, 1893; Bohr, 1909]. Subsequent demonstration of the high diffusion coefficient of carbon dioxide [Krogh, 1919] indicated that passive exchange of carbon dioxide should be ample to provide for its elimination from the foetus. Huggett's "differential tonometry" [1927] pointed to a passive diffusion of  $\text{CO}_2$  across the placenta of the goat, but the data given are few and not entirely conclusive.

Granting for the moment the probability that the elimination of  $\text{CO}_2$  from the mammalian embryo does not involve secretory work, there remains a second problem with regard to the avoidance of acidosis in the embryo. It is well known that the pregnant female is relatively acidotic and that in late pregnancy a degree of acidity is attained which would be considered dangerous in a non-pregnant subject [Williamson, 1923]. If there is always a  $\text{CO}_2$  gradient from the foetus to the mother, the foetus might be expected to show an even more pronounced acidosis.

Material for the study of these questions was available at Cambridge during the investigation of the foetal oxygen relations in the goat [Barcroft, 1933], and the data presented here were obtained from the bloods of the animals used in the experiments on oxygen exchange.

### METHODS.

The anaesthesia, operative technique and collection of the blood in these goat experiments have been described in detail by Barcroft [1933]. All blood was drawn into syringes containing oxalate and

fluoride solution which was in tonometric equilibrium with room air; the oxalate-fluoride solution was 10 p.c. of the final volume in the syringe.

Determinations of  $\text{CO}_2$  and  $\text{O}_2$  contents of the bloods were made in duplicate with the van Slyke apparatus immediately after drawing. A second blood portion was equilibrated with room air for the determination of the oxygen capacity, and the rest of the blood stored in a tonometer with a large gas phase in which the partial pressures of  $\text{CO}_2$  and  $\text{O}_2$  were each about 50 mm. Hg. Blood samples from this tonometer were then equilibrated at  $37^\circ \text{C}$ . in the new Barcroft tonometers [Barcroft, 1934] in gas mixtures (calculated to give about 50 p.c. oxygen saturation) over the general range 30–60 mm. partial pressure of  $\text{CO}_2$ . The blood gas analyses were done in duplicate on 0.2 c.c. portions using the standard van Slyke procedure. At the end of the day a second determination of oxygen capacity was made in most cases; these showed little or no change.

In a few cases blood drawn directly from the animal was analysed for carbon dioxide tension by means of a modification of Krogh's [1908] bubble method.

#### CARBON DIOXIDE CONTENTS AND TENSIONS.

In the earlier stages of pregnancy, foetal blood sufficient for study of the  $\text{CO}_2$  relations was not obtained; however, in two cases carbon dioxide tensions were determined by the bubble technique on blood from the maternal jugular vein of the anesthetized goat after laparotomy. Tensions of 41 and 48 mm. Hg were found; a later determination on the second goat gave a  $\text{CO}_2$  partial pressure of 53 mm. Hg with indication of slight anoxæmia after 85 min. of profound anesthesia and very extensive operative manipulation. These results indicated that maternal  $\text{CO}_2$  elimination was well maintained under the experimental procedure.

In two other cases bubble determinations were successful with both maternal and foetal bloods (from the uterine and umbilical veins respectively). The results are given in Table I; in both cases the carbon dioxide tension on the foetal side of the placenta was slightly higher than on the maternal side.

The other four sets of figures in Table I were obtained from values for  $\text{CO}_2$  contents and the individual  $\text{CO}_2$  dissociation curves for the same bloods; the method of obtaining these figures will be apparent from an inspection of Figs. 1–4. The six cases show clearly that under the

TABLE I. CO<sub>2</sub> tensions in the blood as drawn from the animal. Values in mm. Hg.

Date	No.	Days of pregnancy	Maternal	Fœtal
April 18	8*	85	45	47
May 1	10*	101	46	52
May 5	11†	106	40·8	40·5
May 12	12†	113	41·5	48·5
May 22	14†	126	38·3	51·5
May 31	16†	(105)‡	44·7	44·5

\* Determined by bubble analysis.

† Determined from CO<sub>2</sub> content and CO<sub>2</sub> dissociation curve for the same blood.

‡ Approximate, calculated from weight of fœtus.

experimental conditions CO<sub>2</sub> elimination from the fœtus is effectively maintained without the appearance of any secretory mechanism.

The gas contents of the fœtal blood as drawn from the animals may be compared with values obtained by Huggett [1927] and Cohnstein and Zuntz [1884] for goats.

TABLE II. Mean gas contents of fœtal arterial blood (from the umbilical vein) as drawn from the animal.

Reference	p.c. O <sub>2</sub> saturation	O <sub>2</sub> , vol. p.c.	CO <sub>2</sub> , vol. p.c.
Present determinations	64·1	8·27	47·0
Huggett [1927]	45·0	7·96	29·9
Cohnstein and Zuntz [1884]	—	6·3	40·5

#### THE ALKALI RESERVE.

The carbon dioxide dissociation curves for the fœtal and maternal bloods are shown in Figs. 1-4; they show uniformly a markedly higher alkali reserve in the fœtal bloods than in the respective maternal bloods. That this is not due simply to the smaller cell volumes in the fœtal bloods is shown by the lines for the plasmas (solid lines in the figures) which were calculated from the oxygen capacities and the whole-blood dissociation curves by means of the line charts of van Slyke and Sendroy [1928, see Peters and van Slyke, 1932, p. 289].

The propriety of the use of van Slyke's line charts for these calculations is shown in Table III by the agreement between cell volumes calculated in this way and the cell volumes observed with the hæmatocrit<sup>1</sup>.

The average fœtal whole blood contained 22 p.c. more CO<sub>2</sub> than the corresponding maternal whole blood at the same CO<sub>2</sub> tension; the fœtal plasma averaged 18·6 p.c. higher in CO<sub>2</sub> capacity than the maternal

<sup>1</sup> By the kindness of Dr A. St G. Huggett.

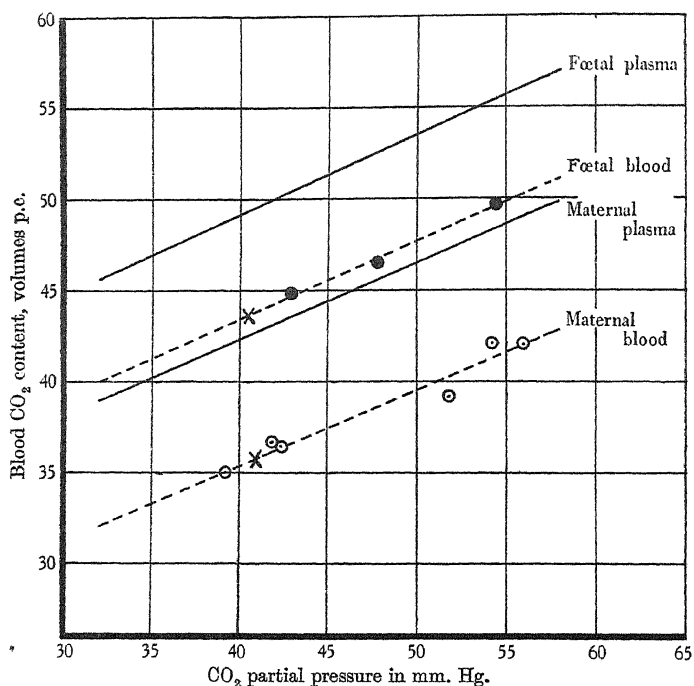


Fig. 1. Carbon dioxide dissociation curves for foetal and maternal whole bloods with calculated lines for the plasmas. Partial pressure of oxygen 47 mm. Hg. throughout. The crosses show the  $\text{CO}_2$  contents of the bloods as drawn from the animal. Goat No. 11, May 5, 106 days' pregnant.

TABLE III. Cell volumes calculated from oxygen capacity and comparison with hæmatocrit values.

Date	Goat No.	Blood	Cell vol. p.c. of whole blood	
			Calculated	Observed
April 18	8	Foetal	38.4	40
May 1	10	Foetal	23.1	22
May 5	11	{ Maternal	39.2	
		{ Foetal	27.0	
May 12	12	{ Maternal	37.1	
		{ Foetal	31.7	32
May 22	14	{ Maternal	40.2	
		{ Foetal	27.8	29
May 31	16	{ Maternal	27.8	
		{ Foetal	25.0	
May 18	Control 13		44.2	
May 16	Control C <sub>3</sub>	{ Before anaesthesia	28.1	
		{ 15 min. "	33.0	
		{ 75 min. "	35.0	
May 29	Control	{ Before anaesthesia	20.8	
		{ 90 min. "	24.9	

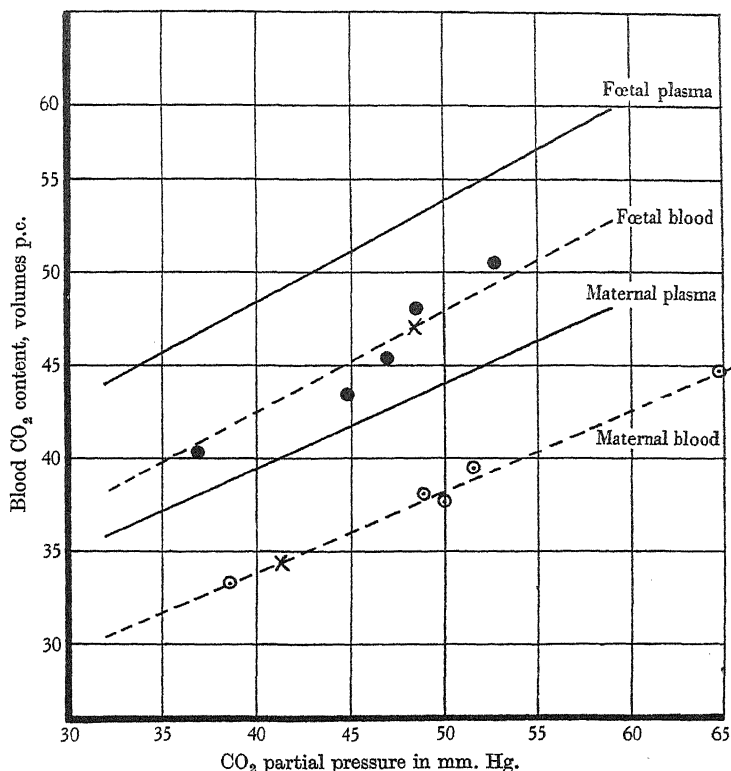


Fig. 2. Carbon dioxide dissociation curves for foetal and maternal whole bloods with calculated lines for the plasmas. Partial pressure of oxygen 43–48 mm. Hg. The crosses show CO<sub>2</sub> contents as drawn. Goat No. 12, May 12, 113 days' pregnant.

TABLE IV. Blood hydrogen ion concentrations at 37°.

Date	Goat No.	Blood	pH calculated	pH observed*
May 5	11	Maternal	7.26	7.26
		Foetal	7.34	7.38
May 12	12	Maternal	7.22	7.25
		Foetal	7.30	7.31
May 22	14	Maternal	7.23	7.14†
		Foetal	7.27	7.30
May 31	16	Maternal	7.33	7.33
		Foetal	7.35	7.34
May 16	Control C <sub>3</sub>	Before anaesthesia	7.37	
		15 min. "	7.31	
		75 min. "	7.35	
May 18	Control 13		7.38	
May 29	Control	Before anaesthesia	7.50	
		90 min. "	7.41	

\* Values obtained with the glass electrode and quoted here by the kindness of Dr R. E. Havard and Mrs S. Dickinson.

† May be erroneous.

plasmas. These figures indicate that, in spite of the foetal-maternal  $\text{CO}_2$  gradient, the foetus would not be more acid than the mother, and might even be expected to be less acid by virtue of the superior alkaline reserve of its blood.

This, in fact, was found to be the case; in every experiment the foetal blood was less acid than that of the mother. In Table IV the hydrogen

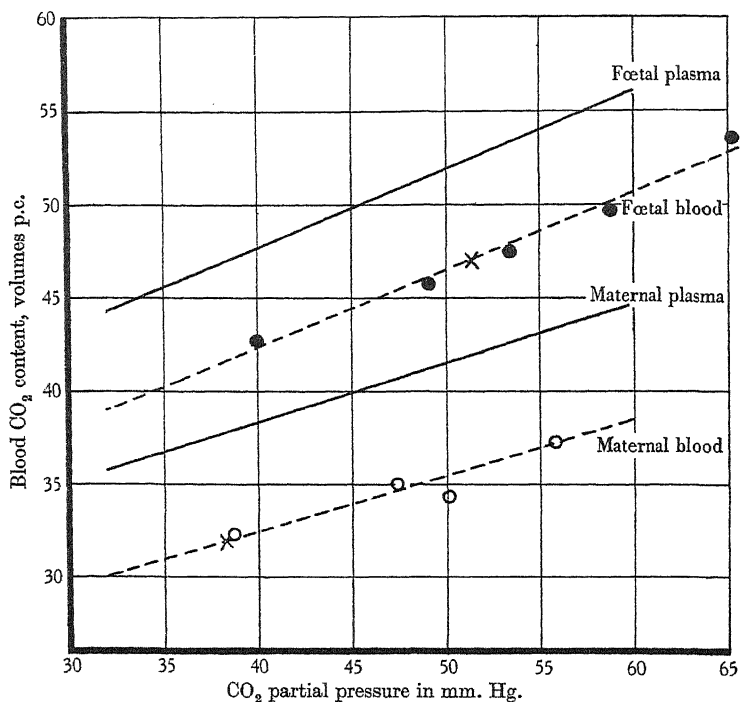


Fig. 3. Carbon dioxide dissociation curves for foetal and maternal whole bloods with calculated lines for the plasmas. Partial pressure of oxygen 43–48 mm. Hg. The crosses show  $\text{CO}_2$  contents as drawn. Goat No. 14, May 22, 126 days' pregnant.

ion concentrations are given for the experimental animals as well as for three non-pregnant female goats. The calculated  $p\text{H}$  values were obtained by the use of the Henderson-Hasselbalch equation:

$$p\text{H} = p\text{k} + \log [\text{BHC}\text{O}_3] - \log [\text{H}_2\text{CO}_3],$$

taking  $p\text{k} = 6.10$  [Hastings, Sendroy and van Slyke, 1928]. For this purpose the line charts of van Slyke and Sendroy given by Peters and van Slyke [1932, pp. 289, 294] were used in most cases.



The excellent agreement between the calculated values and the values obtained by the glass electrode is a triumph for the Henderson-Hasselbalch equation under severe conditions, and warrants more extensive use of this calculation with hitherto uninvestigated bloods.

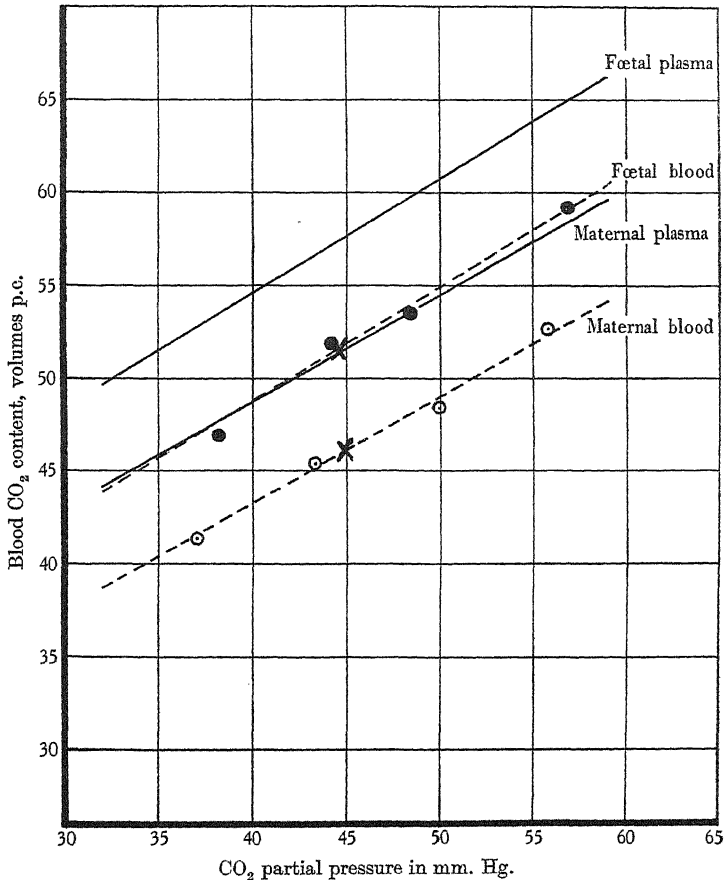


Fig. 4. Carbon dioxide dissociation curves for foetal and maternal whole bloods with calculated lines for the plasmas. Partial pressure of oxygen 30 mm. throughout. The crosses show CO<sub>2</sub> contents as drawn. Goat No. 16, May 31, about 110 days' pregnant.

#### DISCUSSION.

The present series of experiments reveals a nicely adjusted system which provides for elimination of foetal carbon dioxide without requiring secretory work and at the same time safeguards the hydrogen ion con-

centration of the foetal blood. This is not attained, however, without some sacrifice—but the sacrifice is on the part of the mother. Both in alkali reserve and in *pH* the maternal blood alters very much from the normal goat blood, but the foetus is protected and its blood is much more nearly normal than the mother's. This is brought out in Table V in which average values for whole blood are tabulated.

TABLE V. Average value for anaesthetized animals.

	Normal goat	Foetal blood	Maternal blood
CO <sub>2</sub> capacity*	51	47	38
<i>pH</i>	7.38	7.32	7.26

\* At half-saturation with oxygen and partial pressure of CO<sub>2</sub>=45 mm. Hg. Values are in volumes p.c. for whole blood.

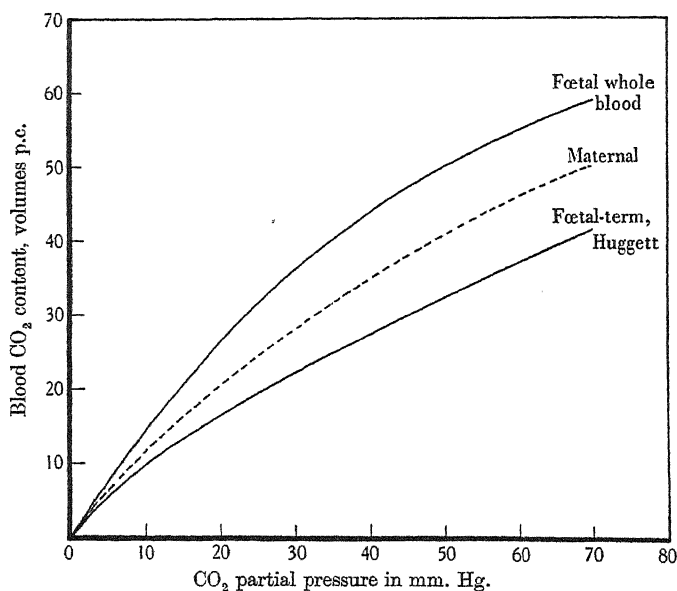


Fig. 5. Complete CO<sub>2</sub> dissociation curves for whole blood. Comparison of average foetal and maternal bloods at 3½ months' pregnancy and foetal blood at term as calculated from Huggett [1927]. All values for oxygen partial pressure 47 mm. Hg.

In the course of foetal development there seems to be a general depletion of the alkali reserve; Williamson [1923] observed a continuous fall in the CO<sub>2</sub> capacity of the blood of pregnant women with the advance of pregnancy. For the goat, figures obtained here for 3½ months' pregnancy may be compared with values calculated from Huggett's data for the foetal blood at or very near term (Fig. 5). Even allowing for possible

differences in states of nutrition, etc., in the two sets of animals, there is a much lower alkali reserve at term than (roughly) six weeks earlier. It is of interest to recall that Zangemeister and Meissl [1903] observed a progressive decrease in the osmotic pressure of the blood of women in pregnancy.

The gas transport properties of foetal blood as indicated in the work here at Cambridge are compared in Table VI with the values calculated from Huggett. The goat foetus at term shows a much higher O<sub>2</sub> capacity, but a much lower CO<sub>2</sub> capacity than the 3½-month foetus.

TABLE VI. Plasma CO<sub>2</sub> capacity of foetal bloods as indicated by CO<sub>2</sub> content at a CO<sub>2</sub> partial pressure of 45 mm., when the partial pressure of oxygen is about 43 mm. Hg.

Fœtuses 100–130 days old. Present determinations		Fœtuses at or near term. Calculated from Huggett [1927]	
O <sub>2</sub> capacity vol. p.c.	Plasma CO <sub>2</sub> capacity vol. p.c.	O <sub>2</sub> capacity vol. p.c.	Plasma CO <sub>2</sub> capacity* vol. p.c.
11.7	57.5	15.5	27.4
12.4	51.3	17	30.2
12.9	49.8	18	44.5
14.1	51.1	19	37.4
—	—	19	48.4
Means 12.8	52.4	17.7	37.6

\* Calculated from partial dissociation curves constructed from Huggett's tonometric data, corrected to O<sub>2</sub> partial pressure of 45 mm. from Huggett's figures for the effect of oxygen on the CO<sub>2</sub> capacity of the blood in his experiments. The transformation from whole blood to blood plasma was made by means of the line chart given by van Slyke and Sendroy [1928].

Unfortunately, Huggett did not collect data from which a comparison of foetal and maternal alkali reserves may be made. In fourteen cases of normal pregnancy, Losee and van Slyke [1917] found maternal plasma CO<sub>2</sub> capacity below the average for non-pregnant normals in all cases, and in ten cases the values were below what is regarded as the "minimum normal." They drew blood from four infants 2–3 days after birth and obtained an average CO<sub>2</sub> capacity of 53 volumes p.c., while the corresponding mothers averaged 50.

Williamson [1923] found the average CO<sub>2</sub> capacity of blood of infants at birth to be 10 p.c. higher than the average for their mothers. He observed that the alkali reserve of the mothers' blood rapidly rises after parturition and is back almost to normal within 10 days. Weissmann-Netter [1925] found an even greater superiority in CO<sub>2</sub> capacity of the blood of normally delivered new-born infants; similar results were obtained from foetal and maternal bloods in four Cæsarian deliveries [Levy-Solal, Weissmann-Netter and Dalsace, 1926].

Opposed to this body of evidence are the results obtained by Bell,

Cunningham, Jowett, Millet and Brooks [1928] who studied two Caesarian section deliveries and a few cases of normal delivery; they found the  $\text{CO}_2$  capacity of the foetal whole blood to average about 5 p.c. less than the maternal blood, and believed this to be due to the high lactic acid level (average 68 mg./100 c.c.) found to characterize the foetal blood.

There is little evidence as to the chemical substances responsible for the difference in physiological buffering power between the maternal and foetal bloods. Unfortunately, the analytical data in the literature in most cases report simply concentrations of the various substances in the whole blood; in view of the pronounced differences in the cell volumes of maternal and foetal bloods, comparison is difficult.

In man, at least, there is practical identity between foetal and maternal blood in respect to total osmotic pressure and chloride, while total protein nitrogen is slightly higher in the maternal blood [Needham, 1931, 3, 1531-3; Fñth and Wirz, 1929].

In the goat Albano [1903] reported the foetal blood to have about 4 p.c. higher osmotic pressure than the maternal blood, but Albano's similar statement with regard to the cow is opposed by the studies of Ubbels [1901] and Grünbaum [1904] who found the same osmotic pressure in both foetal and maternal blood.

All investigators [see Needham, 1931, p. 1531] agree that the concentrations of calcium and inorganic phosphorus are higher in foetal blood than in maternal blood (in man, dog, rabbit, cow and pig). Sodium and potassium have been variously reported as identical, higher in maternal and higher in foetal blood; at first sight the balance of evidence indicates a preponderance of potassium on the foetal side, but when calculations are made on the basis of Na and K in the *plasma*, the values are very nearly equal [Von Oettinger, 1926; Edelstein and Yllpö, 1921; Bakwin and Rivkin, 1927; Krane, 1930].

In the present series of experiments a few measurements of serum refractive index gave slightly higher values for the maternal blood, indicating a probable higher protein concentration in the mother. On the basis of present knowledge it would seem that part of the higher  $\text{CO}_2$  capacity of the foetal blood is due to the excess of phosphates and calcium compounds in it; some other factors, however, must be involved.

#### SUMMARY.

Measurements of carbon dioxide tension in foetal and maternal blood of the goat showed that passive diffusion would suffice for the foetal  $\text{CO}_2$  elimination under the conditions of the experiments.

The foetal blood has a much greater CO<sub>2</sub> capacity than the maternal blood and this is due to a greater alkali reserve in the plasma.

By virtue of this higher alkali reserve the foetal blood is less acid than the maternal blood, in spite of the fact that the foetal CO<sub>2</sub> content is always much larger.

Both in blood pH and in alkali reserve the goat foetus is more nearly normal than its mother.

Prof. J. Barcroft made possible the undertaking of the present study and his stimulating interest secured its completion.

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## A SPECTROSCOPIC METHOD FOR THE STUDY OF HÆMOGLOBIN IN DILUTE SOLUTIONS.

By F. G. HALL.

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*(Received November 17th, 1933.)*

### INTRODUCTION.

THE comparison microspectroscope has been used by several investigators for the study of hæmoglobin [Bürker, 1911]. Krogh [1919] described a method and Krogh and Leitch [1919] used such an instrument to study hæmoglobin in fishes' blood. More recently the microspectroscope has been employed in the study of respiratory pigments by Keilin and Hill. The author is indebted to both Dr Keilin and Mr Hill for suggestions which have led to the use of this instrument as herein described. The following description involves no new use of the microspectroscope. The purpose of this paper is to outline a procedure rather than to describe the use of any particular instrument. Perhaps in this way it may serve others who may be interested in a relatively simple technique for making oxygen dissociation curves on limited quantities of blood.

### THE MICROSPECTROSCOPIC COMPARATOR.

A Zeiss microspectroscopic eyepiece was arranged as shown in Fig. 1. A colorimeter plunger was attached to the tube of the eyepiece. A colorimeter cup was held in a fixed position some centimetres below. Between the fixed cup and the fixed plunger a movable cup ( $C_1$ ) and a movable plunger ( $P_1$ ) could be raised or lowered by means of a rack and pinion. Thus the sum of the depths of solutions in movable and fixed cups was always constant regardless of the position of the movable cup and plunger. A depth of 20 mm. was found a convenient one. Consequently it will be seen that when a solution of fully oxygenated hæmoglobin was placed in one cup and of fully reduced hæmoglobin in the other, one could obtain a spectrum of hæmoglobin representing any degree of oxygenation by

simply varying the relative depth of the solutions in the two cups. The ratio of depths of the two hæmoglobin solutions to one another was read on a vernier scale. A tonometer (*T*) containing the hæmoglobin solution under investigation was placed in front of the side opening of the eyepiece

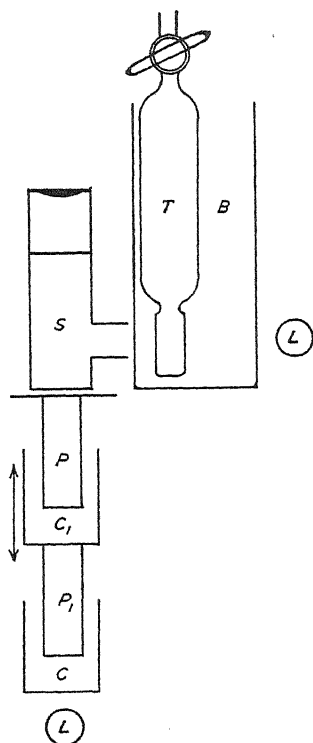


Fig. 1. Diagram to show the relative positions of the spectroscopic eyepiece (*S*), the tonometer (*T*), the colorimeter plungers (*P*, *P*<sub>1</sub>) and the colorimeter cups (*C*, *C*<sub>1</sub>). The tonometer was kept in a glass water bath (*B*). Two lights were fixed in the position (*L*) and their intensity regulated with resistances so that the comparison spectra were of equal intensity.

(Fig. 1). In this manner the two spectra could be matched and the degree of oxygen saturation of the unknown determined.

A nitrogen container (Fig. 2) was constructed by inverting a large bottle and filling it about one-third full with a concentrated solution of sodium hydrosulphite. Nitrogen from a supply tank was bubbled through the hydrosulphite solution and allowed to stand for some hours before using. Samples were withdrawn from time to time and analysed with a

Haldane gas analysis apparatus. No detectable quantity of oxygen was ever found. A manometer (*M*) was arranged to show the pressure within the nitrogen container or within the tonometer as desired. This could be accomplished by manipulation of the stopcock (*S*<sub>2</sub>).

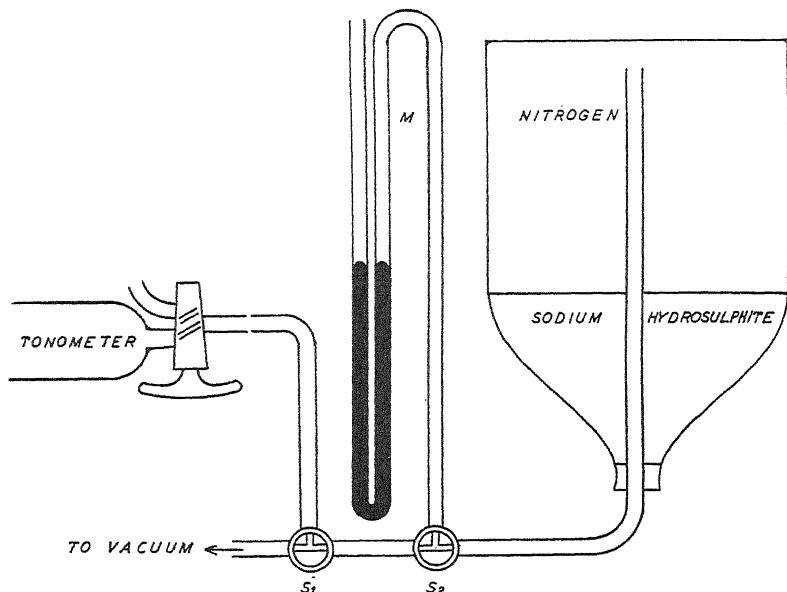


Fig. 2. Diagram to show how tonometers were filled with gas mixtures of known oxygen pressures. Two stopcocks (*S*<sub>1</sub>, *S*<sub>2</sub>) were manipulated so that the tonometer could be alternately evacuated and refilled with nitrogen. Pressures were read on manometer (*M*).

The tonometer was constructed somewhat after that used by Redfield [1930], except that the cell was blown from one piece of glass. The cell was approximately 2 cm. in diameter and 1 cm. deep. The total capacity of the tonometer was about 35 c.c.

#### PROCEDURE.

3 c.c. of a dilute hæmoglobin solution were placed in the tonometer. The tonometer was then evacuated three times, refilling with nitrogen each time. By gentle shaking the dissolved gases could be removed from the solution and the hæmoglobin fully reduced. Fig. 2 shows the position of the stopcocks in relation to the vacuum line, nitrogen supply and tonometer. It was found more convenient to draw the nitrogen from the



manometer by manipulation of a stopcock ( $S_2$ ) than directly from the nitrogen container. After the last evacuation the pressure in the tonometer was left somewhat below atmospheric, and a measured quantity of air was admitted from a gas burette. The temperature of the air in the gas burette and the atmospheric pressure were noted. The tonometer was then shaken for 10 min. in a constant temperature bath. It was then quickly removed to the glass water bath in front of the spectroscope and a reading taken at once.

The standard hæmoglobin solution placed in the colorimeter cups was prepared as follows. Since the depth of the solution in the cups was twice that in the tonometer the hæmoglobin solution was diluted to one-half that in the tonometer. One portion was placed in the lower cup and about 5 mg. of sodium hydrosulphite added. The other portion was saturated with air and placed in the upper cup.

#### CALCULATIONS OF THE OXYGEN TENSIONS.

If  $V_{\text{Ton.}}$  is the capacity of the tonometer in c.c.,  $V_B$  the volume of hæmoglobin in c.c. which has been admitted into the tonometer and freed of dissolved and combined oxygen,  $V_A$  the quantity of air in c.c. introduced into the tonometer at room temperature ( $T_1$ ) and atmospheric pressure ( $P$ ) in mm. of Hg, and  $V_{O_2(c+d)}$  the c.c. of oxygen which is combined with hæmoglobin plus that which is physically dissolved in the hæmoglobin solution after equilibration at temperature  $T_2$ , then the partial pressure of oxygen ( $pp$ ) in mm. of Hg will be:

$$pp = \frac{0.2094 V_A - V_{O_2(c+d)}}{V_{\text{Ton.}} - V_B} \times \frac{T_2}{T_1} \times P.$$

Since the quantity of physically dissolved and combined oxygen is small,  $V_{O_2(c+d)}$  may be approximated without significant change in values for oxygen tensions.

It was thought desirable to compare the spectroscopic method with the van Slyke-Neill manometric method using similar samples of blood. Hæmoglobin from sheep blood was prepared free from stroma protein by the use of ether and salt as described by Adair [1925] and kept in a  $M/15$  phosphate buffer solution of pH 6.8. One portion was diluted fifty times with the phosphate buffer solution. A sample of whole blood after having been hæmolysed with a minimal quantity of distilled water was diluted fifty times with the phosphate buffer solution of pH 6.8. The concentrated hæmoglobin solution was used for the van Slyke technique and equilibrated in Barcroft tonometers. The two

diluted samples treated in a similar manner described above were analysed with the spectroscopic comparator. Three dissociation curves thus obtained are shown in Fig. 3. The two methods seem to check satisfactorily

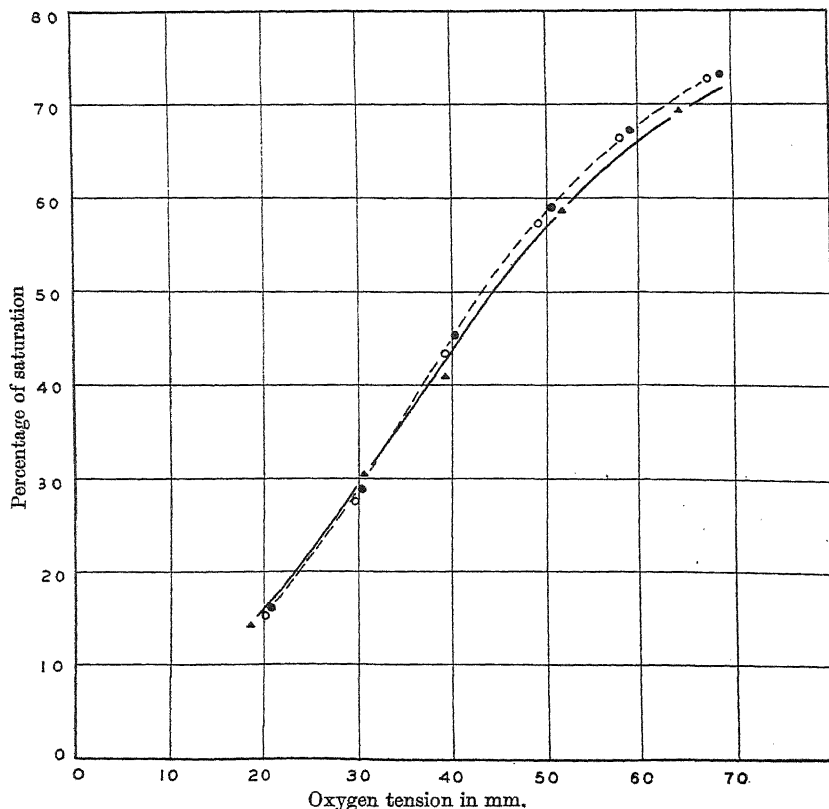


Fig. 3. Three dissociation curves obtained by three different methods. Sheep blood at pH 6.8 was used.

- ▲ represent analyses made with a van Slyke manometric apparatus on a hæmoglobin solution prepared by the Adair method.
- represent analyses made with the comparison spectroscope on whole blood hæmolyzed and diluted fifty times with phosphate buffer solution  $M/15$  and pH 6.8.
- represent analyses made with the comparison spectroscope on the hæmoglobin solution prepared by the Adair method and diluted fifty times with the phosphate buffer solution.

with one another. There appears to be no difference in curves obtained with hæmolyzed whole blood and with hæmoglobin prepared by the Adair method.

There are certain limitations to the spectroscopic method. Determinations of oxygen saturation between 0 and 20 p.c. or between 80 and 100 p.c. cannot be made accurately. Also an error is introduced if appreciable methæmoglobin is formed since the absorption bands of methæmoglobin are quite similar to those of oxyhæmoglobin.

The advantages of the method are: a small quantity of blood is required and it is apparently not necessary to prepare a purified hæmoglobin solution. The  $pH$ , temperature, and oxygen tension can be controlled accurately and easily.

#### SUMMARY.

A spectroscopic method is described whereby dissociation curves can be made on small quantities of hæmoglobin.

I wish to thank Prof. Barcroft and Mr Adair for laboratory facilities and for many helpful suggestions.

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PROCEEDINGS  
OF THE  
PHYSIOLOGICAL SOCIETY,  
*November 18, 1933.*

**A sensitive physical test for adrenaline.** By J. H. GADDUM  
and H. SCHILD.

Paget [1930] and Barker, Eastland and Evers [1932] have noticed that adrenaline, in the presence of alkali and various other reagents, becomes fluorescent. Adrenaline, oxygen, and alkali are, however, the only reagents which are essential for the effect. This reaction can be used as the basis of a test for adrenaline which is more sensitive than other known chemical tests, and in certain respects unexpectedly specific.

In our experiments the solutions were contained in small quartz tubes, with a capacity of about 1 c.c., and illuminated horizontally by light, from a mercury vapour lamp, filtered through a glass which absorbed practically all visible rays. The intensity of the fluorescence in two similar tubes, containing different solutions, was compared by observing them directly from above.

In faintly acid solutions adrenaline showed no fluorescence, but if 0.1 c.c. of 5 N NaOH was added to 1 c.c. of adrenaline solution, a bright apple-green fluorescence appeared, which lasted for about a minute with low concentrations and for several minutes with higher concentrations. The fluorescence lasted longer when oxygen was partially excluded, but did not appear at all in the complete absence of oxygen. A green colour definitely different from the pale blue of the distilled water or diluted NaHO was seen when the concentration of adrenaline was only  $10^{-8}$ . With double the concentration of adrenaline the effect was obviously greater.

Various substances chemically related to adrenaline were also tested. None of these gave a colour nearly so bright as that given by adrenaline in the same dilution, but in certain cases a fainter green fluorescence did develop on the addition of alkali. The intensity of this was estimated by comparing solutions of different strengths with adrenaline solutions, until the intensities of light in the two tubes were about equal. The comparison was made in every case 20 sec. after the addition of alkali. In the following list of substances tested, the figures give the intensity of

the fluorescence, estimated in this way, as a percentage of that due to adrenaline: "Dopa," 3 p.c.; noradrenaline, 2 p.c.; epinine, < 2 p.c.; catechol, 0.1 p.c.; tyramine and ephedrine, nil. The duration of the fluorescence due to these different substances was inversely related to its intensity.

When we attempted to apply this test to blood serum, we found that the fluorescence due to low concentrations of added adrenaline was obscured by blue fluorescence due to other substances. We have not been able to discover a method of completely removing these other substances. Deproteinization by means of trichloroacetic acid or metaphosphoric acid was found to remove added adrenaline, when this was present in low concentrations. It is possible, however, that this test might be used to demonstrate the liberation of adrenaline from tissues into other simpler liquids such as Ringer's solution.

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#### **Nomenclature of fibres in the autonomic system and their effects.** By H. H. DALE.

Evidence has been rapidly accumulating in favour of the view that the effects of nervous impulses in the postganglionic fibres of the autonomic system are chemically transmitted, by substances liberated in relation to their endings, and directly acting on the effector cells. The conception was first put forward by Elliott, in 1904, with regard to sympathetic nerve endings and adrenaline. Really convincing experimental evidence in its favour, for both parasympathetic and sympathetic nerve impulses, was first obtained by O. Loewi, in 1921, and in recent years it has rapidly multiplied. My own earlier observations [1914] on acetylcholine had indicated a substance of this kind as a likely candidate for the function of transmitting the effects of impulses in peripheral parasympathetic fibres; and the evidence in recent years has all tended to increase the probability that this choline ester is the actual parasympathetic transmitter. We have as yet, however, no direct chemical evidence as to the nature of the peripheral transmitter for the other main division of the autonomic system (Cannon's "sympathin"), though physiological evidence indicates a close relation, at least, to adrenaline. Moreover, the correspondence between anatomical origin and functional chemistry is not exact. There are several

examples—*e.g.* the production of sweat secretion, sympathetic vasodilatation and paradoxical contracture in the dog's lips—of effects produced by fibres which are anatomically sympathetic, but apparently transmitted by something like acetylcholine, rather than by something like adrenaline. A further complication arises from the new fact, communicated to-day by Feldberg and Gaddum, that chemical transmission occurs at synapses in a sympathetic ganglion, and that the transmitter, even though the preganglionic fibres are anatomically sympathetic, is a substance indistinguishable from that which transmits the effects of postganglionic parasympathetic impulses. The same point arises in the account by Feldberg, Minz and Tsudzimura of chemical transmission from preganglionic sympathetic fibres to cells of the suprarenal medulla. Both these observations bring into the physiological picture the other aspect of the action of acetylcholine, which I called "nicotine-like."

To avoid elaborate periphrasis, and to promote clear ideas, we seem to need words which will briefly indicate action by two kinds of chemical transmission, due in the one case to some substance like adrenaline, in the other case to a substance like acetylcholine, so that we may distinguish between chemical function and anatomical origin. I suggest the words "adrenergic" and "cholinergic," respectively, for use in this sense. We can then say that postganglionic parasympathetic fibres are predominantly, and perhaps entirely, "cholinergic," and that postganglionic sympathetic fibres are predominantly, though not entirely, "adrenergic," while some, and probably all, of the preganglionic fibres of the whole autonomic system are "cholinergic." I think such a usage would assist clear thinking, without committing us to precise chemical identifications, which may be long in coming.

**Acclimatization (?) of animals to 0.3 p.c. carbon monoxide in the inspired air.** By J. ARGYLL CAMPBELL.

These observations form the continuation of a research to determine the ill-effects, if any, of breathing CO as present in garages, etc., for prolonged periods.

Some young mice and rabbits exposed to CO in the air in gradually increasing concentrations can maintain body growth when inhaling as much as 0.3 p.c. Indeed in mice there is evidence of increased growth compared with the controls, the body weight being increased and the heart revealing hypertrophy. The latter was noted also in some previous experiments. The improved body growth cannot be regarded as a proof

of acclimatization, because the mice exposed to the poisonous gas were not fertile, whereas the controls, similarly treated as regards food, surroundings, etc., were fertile.

Fenn and Cobb [1932] observed that *in vitro* frog's skeletal muscle consumed CO—1 c.mm. per g. per min. at 22° C.—when the gas was present in 80 p.c. concentration. In the present research there was no evidence that the whole animal consumes such a material quantity of CO when breathing 0.3 p.c. for long periods. If, however, we assume that the consumption of the poison by muscle is proportional to the concentration—which perhaps seems doubtful for living tissue—then from Fenn's figures we would expect a rabbit of 2 kg. (muscle 40 p.c.) to consume only about 14 c.c. in 24 hours when exposed to 0.3 p.c. There was no evidence of this quantity being used, but it is too small a quantity to be estimated very accurately (Haldane combustion chamber, large apparatus) under the conditions of the experiment, the respiratory chamber for the animal being about 150 litres gas capacity. In these experiments it was necessary to starve the animal for 2 days to exclude formation of other combustible gases, *e.g.* hydrogen, etc., from the food in the intestines.

The tension of CO in rabbit's tissues was also estimated by establishing gas depots in the abdominal cavity and under the skin. When the animal had breathed 0.3 p.c. CO for prolonged periods, the tension of CO in the tissues was about 0.15 p.c., the analysis of the gas being conducted in the large Haldane analyser. These results give some support to previous work [Campbell, 1929] when the Hartridge spectroscope was used for estimation of CO; in the latter case the tension found was somewhat lower.

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#### **The chemical transmitter at synapses in a sympathetic ganglion.** By W. FELDBERG and J. H. GADDUM.

Kibjakow [1933] perfused the superior cervical ganglion of a cat with Locke-Ringer solution, and found that stimulation of the cervical sympathetic nerve caused the appearance in the outflowing fluid of a substance which, when injected into the fluid flowing to another ganglion, caused a contraction of the nictitating membrane. Chang and Gaddum [1933] found that extracts of the sympathetic chain of a horse contained



comparatively large amounts of a substance identified pharmacologically as acetylcholine, and suggested that the active principle detected by Kibjakow might also be acetylcholine. This view was strengthened by the discovery that stimulation of (preganglionic) sympathetic fibres to the suprarenal medulla caused the liberation of a choline ester [Feldberg, Minz and Tsudzimura, 1933].

In order to test this theory we have perfused the superior cervical ganglia of cats with salt solutions, and applied various pharmacological tests to the outflowing fluid. In the absence of eserine (physostigmine) no activity was detected, but when eserine was added to the perfusion fluid, in order to inhibit the destruction of acetylcholine, it was found that fluid collected during stimulation of the preganglionic nerve had the following properties:

(1) Like acetylcholine, it had no action on a piece of leech muscle untreated with eserine, but it produced a contraction when the preparation had previously been sensitized to acetylcholine by prolonged immersion in eserine. ("Nicotine action.")

(2) It caused inhibition of a frog's heart isolated by Straub's method. This effect was abolished by atropine. ("Muscarine action.")

(3) It caused a fall of blood-pressure in a cat. This effect was also abolished by atropine. ("Muscarine action.")

The solutions were compared with acetylcholine and a quantitative estimate of the concentration was obtained. It was found that when the same solution was tested simultaneously on the frog's heart, and on the leech or the cat's blood-pressure, these estimates agreed quantitatively with one another. We regard these results as strong confirmation of the view that these solutions contained acetylcholine, in a concentration of between 20 and 70  $\gamma$  per litre. Fluid collected in the absence of stimulation and tested in the same concentrations had none of these actions.

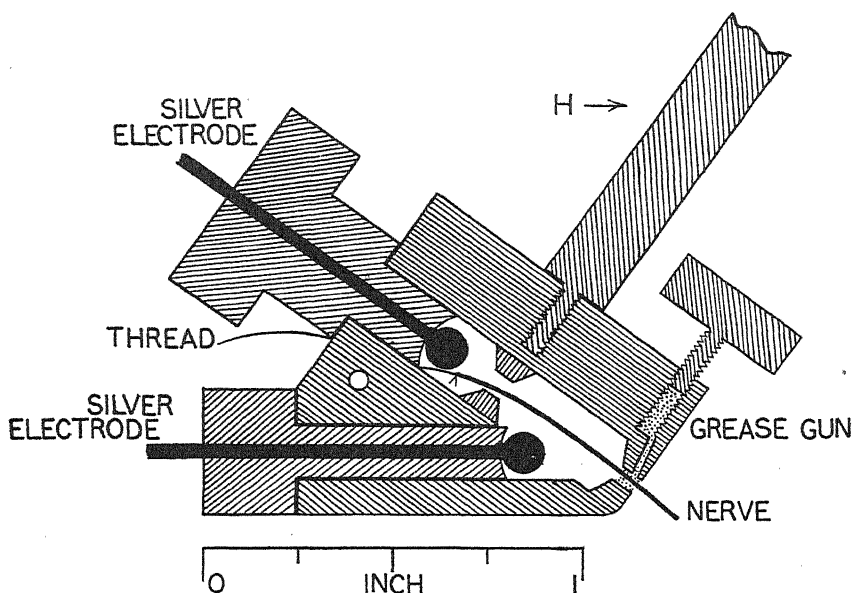
Since choline esters are active stimulants of sympathetic ganglia, it seems probable that the liberation of such a substance forms the normal mechanism by which the effects of preganglionic impulses pass synapses in the ganglion. Our results support the theory that this substance is acetylcholine.

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**A convenient form of fluid electrode.** By L. W. COLLISON.  
(Introduced by H. H. DALE.)

The fluid electrode figured is a modification of that described by Brown and Garry [1932], and has the following features which have been found to give additional convenience in construction and use.



1. The instrument is wedge-shaped in section, and this enables the vulcanite plugs, used in filling the chamber with blood and sealing it, to carry the silver electrodes, for which additional openings are not required. It also enables the instrument to be placed in position in a deep fossa, and to take a relatively small length of dissected nerve.

2. The brass holding-rod (*H*) enables the electrode to be clamped in position.

3. The vulcanite screw functioning as a "grease-gun" greatly facilitates the sealing with vaseline of the hole through which the nerve is drawn into the chamber. The sealing and filling of the electrode are thus normally and easily effected after the apparatus has been fixed in position.

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**The mechanism of the nervous discharge of adrenaline.**

By W. FELDBERG, B. MINZ and H. TSUDZIMURA. (Introduced by H. H. DALE.)

Evidence for the peripheral chemical transmission of the effects of autonomic nerves, both sympathetic and parasympathetic, has accumulated in recent years. The question presented itself whether the impulses in (preganglionic) splanchnic fibres, which cause output of adrenaline from the cells of the suprarenal medulla, produce this effect also by a chemical transmission, and, if so, whether the transmitter is of the sympathetic (adrenaline) type, or of the parasympathetic (acetylcholine) type. We have been able to find evidence that a chemical transmission occurs in this case, and that the transmitting agent, directly affecting the medullary cells, has all the properties of acetylcholine, so far as these can be tested.

1. The body wall of the leech treated with dilute eserine is an extremely sensitive and specific reagent for acetylcholine, contracting in response to dilutions as high as  $1\gamma$  per litre [Fühner, Minz]. It is, on the other hand, quite insensitive to adrenaline, and practically so to the constituents of normal mammalian blood. If eserine is injected into a dog or cat and the splanchnic nerve is stimulated, the blood leaving the suprarenal vein acquires during the stimulation, but only then, a stimulant action on the leech preparation, corresponding to that of acetylcholine in dilutions of  $10-100\gamma$  per litre. The active substance disappears rather quickly from the shed blood, but persists if additional eserine is mixed with it. It has, therefore, the properties of an unstable choline ester.

2. In an eviscerated cat under eserine, the adrenaline rise of blood-pressure caused by stimulation of the splanchnic nerve is preceded by a fall, which a small dose of atropine prevents. If an excess of adrenaline has already been put into circulation, by injecting 2-3 mg. before the splanchnic is stimulated, the atropine-sensitive fall is the only effect of the stimulation.

3. Acetylcholine-like effects, produced by splanchnic stimulation in an eviscerated cat under eserine, can be demonstrated on a distant organ. If the effect of adrenaline on the submaxillary gland is further excluded by injecting 2-3 mg. of ergotoxine, stimulation of the splanchnic nerve causes secretion of saliva, which atropine again abolishes.

4. In an eviscerated cat under atropine, eserine increases the output of adrenaline in response to splanchnic stimulation, as measured by the

rise of blood-pressure. This indicates that the output of adrenaline is due to the liberation of a substance which eserine protects—*i.e.* a choline ester.

5. Acetylcholine itself is a very potent stimulant of adrenaline output from the suprarenal medulla, when it is injected into the arterial stream on its way to the gland. The action is due mainly to the "nicotine" aspect of its action, being almost abolished by large doses of nicotine, which, similarly, almost abolish the adrenaline output in response to splanchnic stimulation. In both cases nicotine often leaves a small residue of effect, always when eserine has also been given, and this residue, due to the "muscarine" action of acetylcholine, is abolished by atropine.

The chemical transmitter of splanchnic action on the suprarenal medulla has in all respects the properties of an unstable choline ester with both "nicotine" and "muscarine" actions, of which the former is in this case predominant.

### **The chemical transmitter of effects of the gastric vagus.**

By H. H. DALE and W. FELDBERG.

The fact that the motor effects of the vagi and the pelvic nerves on the muscular walls of the alimentary canal are so resistant to the action of atropine has been an obstacle to the general acceptance of acetylcholine, or any choline ester, as the probable chemical transmitter of these parasympathetic effects. It seemed important, therefore, to attempt to obtain the chemical transmitter of such an effect, so as to examine its properties separately. This has been done in the case of vagus action on the stomach.

In dogs under chloralose the venous flow into the portal vein was restricted to blood from the stomach, by ligature and excision of other organs. Arrangements were made to collect samples of the portal (gastric) blood. The vagi were exposed in the lower thorax and dissected from the oesophagus for stimulation. A large dose of eserine (physostigmine) (1–2 mg. per kg.) and a smaller one (0.5 mg.) of atropine were given intravenously, and the blood was rendered incoagulable with heparin or a substitute. Samples of portal blood were collected before stimulation of the vagus, and during stimulation adequate to produce powerful contraction of the stomach wall. Eserine was added to the samples as collected to the extent of 1 in 50,000. They were tested on:

- (1) the leech body wall, sensitized to acetylcholine with eserine (nicotine action);
- (2) the blood-pressure of a cat under chloralose (muscarine action).

In all cases the effects produced by one blood sample in these two directions could be matched against the same concentration of acetylcholine. When a large eserine injection had been given, the control blood showed activity which matched with about 20 $\gamma$  of acetylcholine (A.C.) per litre. Vagus stimulation then raised the activity about 2½ times, to match with 50 $\gamma$  A.C. per litre. With a smaller dose of eserine the activity of the control blood was below the limit of measurement (not more than 5 $\gamma$  A.C. per litre), while that of the blood taken during stimulation matched with about 20 $\gamma$  A.C. per litre.

The effects on the cat's blood-pressure were always abolished by 0.1 mg. of atropine, and the leech untreated with eserine gave no specific reaction, with dilutions producing maximal contractions in the sensitized preparation. From blood samples collected without added eserine the activity rapidly disappeared.

Apparently the relatively atropine-resistant effect of the vagus on the stomach is transmitted by a substance having all the properties of an unstable, atropine-sensitive choline ester, indistinguishable from those of acetylcholine, so far as they can be tested in the venous blood collected during vagus stimulation.

**Human electro-cardiograms recorded with the cathode ray oscillograph.** By PIERRE RIJLANT. (Introduced by C. Lovatt Evans.) (*Institut Solvay. Physiology—University of Brussels.*)

In previous communications [1931] we have published human electro-cardiograms taken with a cathode ray oscillograph. These records have a general shape different from that of string galvanometer records. Fast and slow waves, not noticed in galvanometer records, exist in the oscillograph records. Similar waves have been noticed in oscillograph records by Reid [1932] and by Groedel [1933]. Matthews [1933] has recorded human electro-cardiograms with a cathode ray oscillograph. His records are not, at first sight, different from string galvanometer records and show neither fast nor slow extra-waves. Matthews suggests that the waves of the oscillograph records might be due to the amplifier rather than to any activity of the heart. As Matthews overlooked the publication of the exact details of the amplifiers we used [1932], he suggested that the distortion might occur in a capacity resistance distorting circuit of short time constant. The exact details of the amplifiers [1932] and the calibration curves [1933] show that there cannot be any noticeable distortion of

the cardiac waves in our records. To introduce distortion the time constant of our amplifier has to be reduced to one-hundredth of its actual value.

Though for these reasons, complete certitude exists as to the real shape of the human electro-cardiogram as recorded with a cathode oscillograph, we have tested out our previous findings, by the use of a battery coupled amplifier, as distortionless as at present possible [1933], giving the same range of amplification as the amplifiers previously used (four valves).

Cathode ray oscillograms of the human heart taken by this method have exactly the same general shape as the curves recorded with a resistance capacity coupled amplifier of long time constant. The records clearly show the presence of fast waves originating in the sinus and auricles (P 1-P 5), in the His-bundle (*h* 3) and of the slow waves (*h*, S 2, T 2) which Matthews did not see in his records. Inadequate amplification (three valves) seems to be the reason why Matthews's records do not clearly show the fast and slow waves of our records. Record C of Matthews shows clearly, when enlarged, a positive S 2 wave, corresponding to a 100 microvolt potential change (the average value of the S 2 wave in our records).

The site of origin of the waves of the cathode ray oscillogram of the human heart has been traced by using thoracic leads and by comparing the records obtained under these circumstances with the records obtained with the classical leads D I, D II, and D III. Comparison of records of the human electrogram with those of the dog's heart do not show any striking difference as the dog's records show exactly the same slow and fast waves as the human record. In the dog the heart potentials have been led off by thoracic leads and by electrodes directly put on the exposed heart. Both records show the same waves.

The fast and slow waves of the human electro-cardiogram recorded with the cathode ray oscillograph are due to the activity of the heart and not to a modification of the heart's action-potential by conduction through the surrounding tissues.

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**Glycolytic formation of blood lactate.** By C. LOVATT EVANS, FONG-YEN HSU<sup>1</sup> and TAKAO KOSAKA<sup>2</sup>. (*University College, London.*)

It has been shown [Evans *et al.*, 1933] that the heart uses much more of lactate than of sugar from the blood (confirming McGinty); further, that the balance of continual formation of lactic acid by glycolysis in the blood, and its continual removal by the heart (or other tissue) might account for the phenomenon described in the heart-lung preparation by Anrep and Cannan [1923], and in the limb-lung by Eggleton and Evans [1930]. The margin of lactate formation was, however, apparently small, and this is particularly so if the argument be extended to the intact body in explanation of the normal resting blood lactate [Cook and Hurst, 1933].

We have now studied the rate of lactate formation in defibrinated blood perfused through ventilated lungs, and find that glycolysis is two or three times as rapid when blood is so circulated as when oxygenated by circulation *in vitro*. Removal of one lung does not seem to lower the rate of glycolysis if the blood flow through the remaining lung is the same as that previously through both. When the blood which has been passed through the lungs and that which has circulated through the oxygenator are interchanged after a period, the same difference is seen, *i.e.* that now passing through the lungs is the more rapidly glycolysed. In the lung about 80 p.c. of the glucose lost is converted into lactic acid; in the oxygenator the conversion is rather irregular, which may be associated with hæmolysis.

The following is an abstract of an experiment:

Hours from start	Lung circuit mg./100 c.c. blood		Oxygenator circuit mg./100 c.c. blood	
	Glucose	Lactic acid	Glucose	Lactic acid
0-00	126 } 30	71 } 25	142 } 17	65 } 17
1-00	96 } 29	96 } 33	125 } 19	82 } 22
2-00	67 }	129 }	106 }	104 }
2-01	Circuits interchanged			
2-18	85 } 27	126 } 15	78 } 14	129 } 5
3-18	58 } 33	141 } 15	64 } 7	134 } 14
4-18	25 }	156 }	57 }	148 }

<sup>1</sup> China Foundation Fellow.

<sup>2</sup> Rockefeller Foundation Fellow.

In one experiment the lactate in the lung circuit rose at a rate of about 25 mg./100 c.c./hr. until all the blood sugar had disappeared and then fell 5 mg./100 c.c. in the next hour, thus indicating the origin of the lactic acid.

We suggest that the accelerated glycolysis in the lungs gives enough lactic acid to account fully for the phenomena mentioned, and probably for the formation of the resting blood lactate in the intact body. Further investigation is in progress.

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**Duality of the mechanisms of tonus and contraction in striated muscle.** By PIERRE RIJLANT. (Introduced by C. LOVATT EVANS.) (*Institut Solvay. Physiology—University of Brussels.*)

Two types of action currents can be led off by needle electrodes from a normally innervated striated muscle: fast waves of high potential, corresponding to contraction; much slower and smaller waves corresponding to tonic activity. The average amplitude of the "tonic" waves is one-third of the amplitude of the "contraction" waves in single motor units. The duration of the tonic waves is twice that of the contraction waves. The average frequencies of the contraction waves (30 per sec.) are higher than those of the tonic waves (10 per sec.), but the inferior limit of both is about the same (5 waves per sec. in mammals).

During the hypnotic sleep in mammals, the contraction waves disappear completely, except for the contraction waves of respiratory muscles, while the tonic waves remain unchanged. Deep hypnotic sleep abolishes the tonic reactions as well as contraction. When given in small amounts, curare completely abolishes the tonic waves while the contraction waves are maintained temporarily.

Two types of action currents can be led off from the central end of a motor nerve. They show the same characteristics as the two types of waves led off from the striated muscle which are related to tonus and contraction.

From human striated muscles (triceps, quadriceps) the same action currents can be led off. The fast contraction waves are frequently



built up by four to ten very fast waves slightly asynchronous. Some outbursts of tonic waves are related to the respiratory cycle. Other rhythmic outbursts of tonic waves, not related to the respiratory cycle, of an average duration of one second, are probably related to the Traube Hering oscillations of blood-pressure.

In the male amphibian, during the mating season, the tonic activities of the striated muscles of the forearm are accompanied by electrical waves of very long duration (100–200 $\sigma$ ). These waves originate in the same fibres which normally are responsible for the tonic waves (10–20 $\sigma$ ). The action currents of the motor nerves of the forearm are not different during the mating season from what they are under normal circumstances.

The change in duration of the tonic waves is only due to the change in the reaction of the striated muscle. The long duration of the tonic waves during the mating season suggests a humoral transmission at the neuro-muscular junction.

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### **The sensitization of leech muscle to barium by eserine.**

By A. VARTIAINEN. (Introduced by H. H. DALE.)

Fühner [1918] found that eserine caused a very remarkable sensitization of the dorsal muscle of a leech to acetylcholine, so that after eserine it reacted to a millionth of the concentration which had been necessary to cause a contraction before eserine. He suggested the theory that this sensitization was due to the inhibition of choline esterase, and recent work has confirmed this view.

Fühner also found that eserine sensitized the leech to barium. This observation is of importance, since it seems to weaken arguments based on the assumption that any effect which is increased by eserine must necessarily be due to a choline ester.

Fühner's experiments with barium and eserine have therefore been repeated. The fact that eserine has some sensitizing action on the response of barium was confirmed, but this sensitization was not so striking as might be supposed from Fühner's papers, and was not comparable with that to acetylcholine. When repeated doses of barium were applied at constant time intervals to an isolated piece of leech muscle, left in contact with the muscle for 3 min. and then washed out, successive doses

produced increasing contractions, so that a certain amount of apparent sensitization occurred even in the absence of eserine. After some time, however, this increase in sensitivity ceased, and successive small doses produced a constant effect. If eserine was now added to the bath it caused some increase in the response to barium, but only to such an extent that the response was now equal to that produced by not more than twice the concentration of barium, before eserine.

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# PROCEEDINGS

## OF THE

# PHYSIOLOGICAL SOCIETY,

*December 16, 1933.*

### Action potentials from the superior cervical ganglion.

By J. C. ECCLES.

Electrodes have been applied to the cat's cervical sympathetic trunk 4 or 5 cm. below the superior cervical ganglion and single induction shocks have been used as stimuli. By means of non-polarizable leads, action potentials so set up in the superior cervical ganglion have been led to an amplifier connected to a Matthews' oscillograph, the earthed lead being on the ganglion and the grid lead on the crushed end of the post-ganglionic trunk. The blood supply of the ganglion and of the cervical sympathetic has not been interfered with, but the vagus and the ganglion nodosum have been removed.

A complex negative wave appears in the ganglion 5–7  $\sigma$  after stimulation of the cervical sympathetic, attaining at 12–17  $\sigma$  after the stimulus a maximum which may be as high as 0.5 mv. There are three components in this wave, the summit being formed by the second component, while the third appears as a small hump late on the descending phase. Finally a fourth negative wave becomes first recognizable 30–40  $\sigma$  after the stimulus, reaches a low maximum at 45–60  $\sigma$  and is still usually present at 70  $\sigma$ . As the strength of the stimulus is gradually weakened, the fourth wave may be the first to disappear, and then the third, second and first waves may disappear in turn. However, the thresholds characteristic of each of these waves are not very sharply differentiated, *e.g.* the fourth wave has been present with strengths of stimuli which were submaximal for the first and second waves.

A study of the pre-ganglionic action potentials shows two waves, one conducting at 20–25 m. per sec., the other at about 15 m. per sec. The first pre-ganglionic impulses therefore arrive at the ganglion 2–2.5  $\sigma$  after the stimulus, so there is a latent period in the ganglion of at least 3  $\sigma$  before the ganglionic action potential appears.

When a second pre-ganglionic stimulus is applied at an interval as short as 3.7  $\sigma$  after a previous stimulus, it gives rise to an action potential

in the ganglion. The latent period of the onset of this potential may be  $1\sigma$  shorter than normal, and the first potential wave rises much more steeply than normal. It is of course superposed on the potential wave set up by the first stimulus. Since the pre-ganglionic conduction time for the volley set up by the second stimulus is increased by about  $1.5\sigma$ , this pre-ganglionic volley must arrive at the ganglion only  $1\sigma$  before it sets up a ganglionic action potential, *i.e.* the latency in the ganglion has been considerably shortened. The second ganglionic wave is also set up by a stimulus applied  $3.7\sigma$  after the first, but its latent period appears increased by an amount equal to the lengthening of the pre-ganglionic conduction time. The third wave is absent at  $3.7\sigma$  interval, but present when the interval was  $5.7\sigma$ , and the fourth wave only appears after stimuli at  $12$ – $15\sigma$  intervals, its maximum being attained at intervals greater than  $32\sigma$ . The first three waves appear normal in size after stimuli applied at intervals of  $12\sigma$  or longer. The threshold and refractory period for the setting up of pre-ganglionic nerve impulses are identical with the values obtained for the ganglionic action potentials.

The various component waves in the ganglionic action potential could be ascribed to pre-ganglionic fibres with conduction times corresponding with the delay in the appearance of the respective waves [cf. Bishop and Heinbecker, 1932], *e.g.* the fourth wave could be due to the C fibres which conduct at 1–2 m. per sec.; but the waves appear, though with a diminished latency, even when the pre-ganglionic trunk is stimulated just proximal to the ganglion, so it seems that the greater part of the delay in the appearance of the second, third and fourth waves must be due to a delay in the ganglion corresponding with that found for the first wave.

When the blood supply to the ganglion is cut off, the latent period of the ganglionic action potential is increased to as much as  $15\sigma$ , the summit being reached as late as  $27\sigma$ , while a second stimulus even as late as  $12\sigma$  after the first fails to set up any ganglionic action potential, though it gives rise to a full-sized pre-ganglionic wave. Bishop and Heinbecker [1932] state that there is an absolutely refractory period of  $20\sigma$  and a synaptic delay of  $10\sigma$  for conduction through the superior cervical ganglion, whereas Brown [1933] finds that both refractory period and synaptic delay are less than  $3\sigma$ . This discrepancy is no doubt due to the fact that Bishop and Heinbecker worked on excised ganglia.

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**The effects of eserine and atropine on the vagal slowing of the heart.** By J. C. ECCLES.

In a previous communication [Brown, Eccles and Hoff, 1932] the inhibitory effect of single vagal volleys on the heart rhythm was described, and the time course of this inhibition was depicted by plotting curves with lengthenings of cycles as ordinates and the corresponding intervals after the vagal stimuli as abscissæ. An initial maximum inhibition is always attained about 0.5 sec. after the vagal stimulus, and is followed by a rapid decline, the inhibitory effect falling to half its maximum in 0.4–0.8 sec. This rapid inhibitory wave is followed by a slower inhibitory wave first perceptible 0.6–1.5 sec. after the vagal stimulus, the maximum being reached 2–2.5 sec. after the stimulus. When the first inhibitory wave is high and long-lasting it obscures the onset and even the summit of this second inhibitory wave, which may appear merely as a hump or flattening on the descending part of the first wave. At the summit of the first wave there may be even more than a doubling of the duration of the cardiac cycle, but the maximum of the second wave is never more than a 10 p.c. lengthening.

As the stimulation of the vagus is progressively made more and more submaximal the height of the first wave declines much more rapidly than the second, and with small vagal effects the second wave may be higher than the first. The inhibitory curve of the left vagus is always identical with a submaximal inhibitory curve of the right vagus. When a second vagal volley is set up (either in the same or other vagus) at any interval after the first, its first wave always adds directly on to the inhibitory curve produced by the first volley, but its second wave is always largely occluded by the second wave set up by the first volley. Tetanic excitation of the nervi accelerantes always diminishes the second wave and may cause it to disappear.

The following possible explanations of the double inhibitory waves have been excluded:

(1) The trough between the two inhibitory waves being due to a transient displacement of the pacemaker to another part of the sino-auricular node.

(2) The trough between the inhibitory waves being due to a transient accelerator action from accelerator fibres in the vagus.

(3) The second wave being due to after-discharge from the post-ganglionic neurones of the vagus.

It seems therefore that the double inhibitory wave is an expression of a double inhibitory effect produced by a single volley of impulses arriving at the terminals of the post-ganglionic fibres of the vagus.

Eserine and atropine have been used to obtain further evidence on the nature of these two inhibitory waves. Eserine given intravenously in doses of  $25\gamma$  to 2-3 kg. cats increases the height of the first wave and approximately halves the rate of its decline. The height of the second wave is similarly affected but to a relatively smaller extent. The maximum effect is attained in 3 min. after the injection and it has completely passed off in 2-3 hours. Doses of eserine several times larger so prolong the first wave that it completely overlaps the second. Atropine in very small doses, *e.g.*  $5\gamma$ , lowers the inhibitory curve so that it becomes identical with the curve produced by submaximal stimulation, *i.e.* the height of the first wave is reduced relatively much more than the height of the second wave. This indicates that the inhibitory processes responsible for the two waves are similarly affected by atropine.

Thus the effects of eserine and atropine indicate that both inhibitory waves are due to the same inhibitory substance, presumably acetylcholine, and the effect of eserine indicates that all phases of the decline of the inhibitory curve are due to the enzymatic hydrolysis of acetylcholine. The concentration of eserine which halves the rate of this hydrolysis corresponds approximately with that found by Matthes [1930] in *in vitro* experiments on the hydrolysis of acetylcholine.

#### REFERENCES.

- Brown, G. L., Eccles, J. C. and Hoff, H. E. (1932). *J. Physiol.* 76, 9 P.  
Matthes, K. (1930). *Ibid.* 70, 338.

#### **Blood gas figures in a case of Fallot's tetralogy.** By M. KREMER. (*Department of Physiology, Middlesex Hospital.*)

J. R., a Welsh miner aged 35, had been noticing increasing blueness during the last two years with marked shortness of breath. There were occasional attacks of giddiness with faintness but no loss of consciousness.

Examination showed slight enlargement of the heart to the left, and this was confirmed by X-ray which showed a "sabot shaped" heart. There was a systolic thrill and murmur in the third left interspace about  $\frac{1}{2}$  in. from the sternal edge. Very marked cyanosis and clubbing of fingers and toes. A diagnosis of Fallot's tetralogy was made.

By means of arterial puncture an attempt was made to calculate the arterio venous shunt. Blood was taken from the femoral artery and it

oxygen content and capacity estimated by means of the Haldane blood gas analysis apparatus.

Duplicate estimations gave the following results: oxygen content = 17.75 c.c. p.c.; oxygen capacity = 34.75 c.c. p.c.; saturation, p.c. = 51.

Further estimations were done after the patient had inhaled pure oxygen for  $\frac{3}{4}$  hour through a nasal catheter. Oxygen content rose to 22.1 c.c. p.c. and percentage saturation to 64 p.c.

The oxygen capacity was also determined by colorimetric and gravimetric estimations of hæmoglobin. The Hb by the Haldane standard was 140 p.c. (= 20 g. Hb = 27 c.c. O<sub>2</sub> p.c.). Gravimetric estimation was 26 g. per 100 c.c. = 34.5 c.c. O<sub>2</sub> p.c. It will be seen that the gasometric estimation agrees closely with the gravimetric but not with the colorimetric, which is probably less reliable at high concentrations of hæmoglobin.

From the above gasometric data the degree of arterio venous shunt may be calculated. Assuming that all the unsaturation is due to shunt, then using the formula of Segall [1928] let  $X$  = venous shunt as from right to left side of circulation in p.c. of total blood flow and  $100 - X$  = p.c. amount of blood entering arterial system from pulmonary veins, then

$$\text{Oxygen in arterial blood} = \frac{(\text{venous oxygen}) X}{100} + \frac{(\text{oxygen in pulmonary veins})(100 - X)}{100}.$$

Following Lundsgaard it may be assumed that the oxygen in pulmonary veins is 95 p.c. of oxygen capacity and that oxygen in mixed venous blood is 5 volumes p.c. less than arterial oxygen content. Substituting these figures we obtain

$$17.75 = \frac{(12.75) X}{100} + \frac{33}{100} (100 - X),$$

which gives a venous shunt of 75 p.c.

An alteration of  $\pm 1$  c.c. in venous oxygen content makes a difference of  $\pm 5$  p.c. in venous shunt.

From the oxygen inhalation experiment it appears, however, that the unsaturation of the arterial blood is partly due to incomplete aeration of the venous blood in the lungs. Using the data of the inhalation experiment the shunt is calculated to be 69 p.c.

This probably represents a true index of the state of affairs existing.

#### REFERENCE.

Segall (1928). *Blumer's Bedside Diagnosis*, 11, 447.





10 c.c. The expired air can be collected from the outlet. The record is unaffected by the movements of the animal other than respiratory. The spirometer has some inertia and the first five or six breaths recorded by it are less deep than the succeeding ones. When breathing is exceedingly shallow, as just prior to respiratory arrest, expiration may be too short to make the contact *E* or too brief to open the valve *A* for a sufficient time to refill the spirometer. This difficulty occurs rarely. I have had the apparatus in use for several months and found it otherwise quite reliable. It is made by C. F. Palmer, Ltd., and I am indebted to Mr E. Ellis for much technical assistance.

**Muscle phosphorus compounds in adrenal insufficiency.** By EINAR LUNDSGAARD and A. T. WILSON. (*Departments of Physiology, University of Copenhagen, and Middlesex Hospital.*)

Muscular weakness, a leading symptom of experimental adrenalectomy and of Addison's disease, has been related to a reported quantitative alteration in the phosphagen content of muscle. [Lang, 1931; Ochoa, 1932.]

In this research muscle specimens were obtained from cats between 5 and 8 days after bilateral adrenalectomy, when various gross symptoms were present. The animals were shot by a "humane killer" (or anaesthetized with "Nembutal"); a portion of muscle, cut out as rapidly as possible from rectus femoris, was dropped into liquid air.

For determination of the various phosphorus compounds, the methods of Lohmann and Jendrassik [1926], and Lohmann [1928] were used.

$P_2O_5$  in the form of inorganic phosphate, phosphagen, adenylyl-pyrophosphate and hexose-phosphate was determined in five doubly adrenalectomized animals and also in five controls, unilaterally adrenalectomized, subjected to a second "dummy" operation, and given the same amount of food as was consumed by the completely adrenalectomized animals. The clinical and muscular condition of the controls was normal.

	Adrenalectomized		Controls
	(Average values in mg. $P_2O_5$ per g. of muscle.)		
Inorganic phosphate	56	} 159	65
Phosphagen	103		90
Pyrophosphate	39		39
Hexose-phosphate	46		60

With one exception, the phosphagen range lay between 107 and 121 mg. per g. in the adrenalectomized animals.

The slight differences in the amount of these substances in the two groups may be due to differences in muscular activity at the time of death.

It seems improbable that the gross muscular weakness of the adrenalectomized animal can be related directly to the small alterations in muscle phosphorus compounds noted.

*Typical protocol.*

*Cat 91.* Male. 3.4 kg. Adrenalectomy in two stages. First symptoms on 3rd day after 2nd operation. Symptoms well marked on 7th day after which the animal was shot.

Blood urea: 215 mg. per 100 c.c. Blood non-protein nitrogen—118 mg. per 100 c.c.

Blood sugar: 42 mg. per 100 c.c. Fixed  $\text{CO}_2$  of blood—34 c.c.  $\text{CO}_2$  p.c.

*Control Cat 82*, unilaterally adrenalectomized and with second "dummy" operation. Clinical condition normal when shot on 7th day.

Blood urea: 31 mg. per 100 c.c.

N.P.N.: 30 mg. per 100 c.c.

Blood sugar: 150 mg. per 100 c.c. (emotional hyperglycæmia).

Fixed  $\text{CO}_2$ : 35 c.c.  $\text{CO}_2$  p.c.

Figures given below are in mg.  $\text{P}_2\text{O}_5$  per g. of muscle.

Adrenalectomized (No. 91)		Control (No. 82)
Inorganic phosphate	62	59
Phosphagen	110	91
Adenyl-pyrophosphate	40	35
Hexose-phosphate	50	65
Total P	262	250

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 Lohmann and Jendrassik (1926). *Biochem. Z.* **178**, 418.  
 Lohmann (1928). *Ibid.* **194**, 306.  
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